Role of PI3Kα and PI3Kβ Signaling in Post-infarction Cardiac Remodeling

by

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Abstract

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide, while ischemic heart disease (IHD) dominates the cause of CVD mortality. Following myocardial infarction (MI), the heart experiences a series of structural and functional changes, termed post-infarction cardiac remodeling, characterized by ventricular dilation, eccentric hypertrophy, inflammation, and fibrotic scar formation. Within the heart, cardiomyocytes and endothelial cells are critical to maintain cardiac function. Moreover, they are essential players in orchestrating the complex post-infarction responses. Cardiomyocytes, especially those in the infarcted region, struggle to survive, while the survived cardiomyocytes undergo hypertrophy attempting to maintain cardiac function. Cardiac endothelial cells can be rapidly activated and undergo angiogenesis in response to myocardial injury, orchestrating cardiac remodeling processes. Attempting to attenuate post-MI adverse remodeling, I evaluated the roles of PI3K isoforms in IHD.

Class I phosphoinositide 3-kinases (PI3K) are conserved lipid kinases that produce the second massager PIP3 which subsequently leads to the activation of various downstream effectors. They contain four isoforms, PI3Kα, PI3Kβ, PI3Kδ, and PI3Kγ. While PI3Kα and PI3Kβ are ubiquitously expressed, PI3Kδ and PI3Kγ are enriched in leukocytes. Previous research has highlighted the pro-survival roles of PI3Kα in tumor progression, leading to the development of PI3Kα inhibitors as a novel anti-cancer therapy. In the heart, PI3Kα is involved in cardiomyocyte hypertrophy, contractility, and post-injury viability. Moreover, PI3Kα in the endothelial cells participates in angiogenic processes. On the contrary, PI3Kβ has been considered to be redundant for decades. Recent studies have suggested the function of PI3Kβ in cell survival, DNA replication, and DNA repair. Nonetheless, little is known about the role of PI3Kα and PI3Kβ in post-MI cardiac remodeling. With the knowledge gaps in mind, I investigated how PI3Kα and

PI3Kβ function in endothelial cells and cardiomyocytes to modulate cardiac function and post-infarct ventricular remodeling.

First, I investigated whether PI3Kα inhibition affects cardiac health and post-MI cardiac healing and remodeling. WT mice with daily and oral PI3Kα inhibitor-BYL719 administration for 10 days showed reduced left ventricular longitudinal strain with normal ejection fraction, weight loss, decreased heart weight, body composition alteration, and prolonged QT interval. BYL719 also aggravated cardiac dysfunction and cardiac remodeling after MI, with increased apoptosis, elevated inflammation, decreased vascular density, and inhibited Akt/GSK3β/eNOS signaling. Genetic inactivation of PI3Kα specifically in cardiomyocytes suggests PI3Kα regulates baseline cardiac function. Either endothelial- or cardiomyocyte-PI3Kα inactivation led to a markedly deterioration of cardiac function after MI. Whilst lack of endothelial PI3Kα suppressed endothelial repair with decreased endothelial survival, proliferation, and vascular density, loss of cardiomyocyte PI3Kα enhanced post-MI cell apoptosis and inhibited hypertrophy. BYL719 suppressed endothelial Akt/eNOS activation, cell viability, proliferation, and angiogenic responses. Moreover, it decreased hypoxia associated Akt activation and cell survival in isolated adult mouse cardiomyocytes. Thus, PI3Kα inhibition is detrimental to post-MI cardiac repair by suppressing endothelial repair, cardiomyocyte survival, and hypertrophic responses.

I then examined the role of endothelial and cardiomyocyte PI3Kβ in post-infarct cardiac remodeling. Loss of endothelial PI3Kβ resulted in marked resistance to infarction with decreased mortality, improved systolic function, preserved microvasculature, and enhanced Akt activation. Cultured endothelial cells with PI3Kβ knockout or inhibition displayed preferential PI3Kα/Akt/eNOS signaling that consequently promoted angiogenesis. In contrast, mice with cardiomyocyte PI3Kβ-deficiency exhibited adverse post-infarct ventricular remodeling with larger infarct size and deteriorated cardiac function, which was due to enhanced susceptibility of cardiomyocytes to ischemia-mediated cell death. Disruption of cardiomyocyte PI3Kβ signaling

compromised nuclear PI3Kβ and phospho-Akt levels, leading to perturbed gene expression and elevated pro-cell death protein levels. This study demonstrates novel, differential, and cell-specific functions of PI3Kβ in the ischemic heart. While the loss of endothelial PI3Kβ activity is cardioprotective, cardiomyocyte PI3Kβ is required for myocardial ischemic recovery.

In summary, the studies have demonstrated the cell type-specific function of PI3Kα and PI3Kβ in cardiac tissue in the healthy and ischemic heart. Manipulation of PI3Kα and/or PI3Kβ has therapeutic potential in IHD. While amplification of cardiac PI3Kα might provide beneficial effects to the ischemic heart by protecting cardiomyocyte from apoptosis and supporting angiogenesis, small-molecule inhibitors targeting PI3Kβ might improve post-MI cardiac repair by enhancing vascular repair. Moreover, with the growing evidence supporting the use of PI3Kα inhibitors in advanced tumors, the concerns of potential cardiotoxicity of chronic treatments should be raised, especially when coinciding with an ischemic event.

Preface

This thesis is an original work by Xueyi Chen. The work presented in the dissertation was mainly carried out in Dr. Gavin Oudit's and Dr. Zamaneh Kassiri's laboratories in the University of Alberta, Edmonton, AB, Canada. Human heart specimens were collected as part of the Human Organ Procurement and Exchange program (HOPE) and Human Explanted Heart Program (HELP). Informed and signed consents were obtained from all participants. The collection of human cardiac tissues and ethics were approved by the Mazankowski Alberta Heart Institute and the Institutional Ethics Committee. All animal experiments were conducted in accordance with the Canadian Council for Animal Care guidelines, with protocols approved by the Animal Care and Use Committee at the University of Alberta.

A version of Chapter 3 of the dissertation will be submitted to a peer-reviewed journal as *Chen X, Zhabyeyev P, Azad AK, Wang W, DesAulniers J, Vanhaesebroeck B, Murray AG, Kassiri Z, Oudit GY. PI3Kα Inhibition Leads to Impaired Cardiac Healing and Adverse Cardiac Remodeling After Myocardial Infarction*. I was responsible for study design, performing the experiments, data collection and analysis, and manuscript writing. Specifically, I conceived and designed experiments, performed all the *in vivo* and *in vitro* experiments (animal treatment and tissue collection, echocardiographic analysis, cardiomyocyte isolation and culture, endothelial cell culture, flow cytometry, all immunofluorescence staining, and all Western blots) except the ones indicated below, acquired and analyzed the data, prepared figures, drew illustrations, and wrote the manuscript. Dr. Zhabyeyev helped with adult murine cardiomyocyte isolation and cyclical mechanical stretching, electrocardiogram on mice, and electrocardiogram analysis. Dr. Azad carried out the angiogenic bead assay. Dr. Wang performed Sham and MI surgery and Lectin intravital perfusion on mice. Ms. DesAulniers took care of the animal breeding. Dr. Murray, Dr. Kassiri, and Dr. Vanhaesebroeck provided important comments to the project. Dr. Oudit was the Corresponding author who involved with concept formation and supervised the project.

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echocardiographic analysis, cardiomyocyte isolation and culture, endothelial cell culture, all immunofluorescence staining, nuclear protein extraction, and all Western blots) except the ones indicated below, acquired and analyzed the data, prepared figures, drew illustrations, and wrote and revised the manuscript. Dr. Zhabyeyev helped with adult murine cardiomyocyte isolation and cyclical mechanical stretching and electrocardiogram experiments. Dr. Azad carried out the angiogenic bead assay. Dr. Wang performed Sham and MI/IR surgery and Lectin intravital perfusion on mice. Dr. Minerath conducted RNA sequencing and following analysis. Ms. DesAulniers took care of the animal breeding and the quantitative TaqMan real-time PCR experiments. Dr. Grueter, Dr. Murray, Dr. Kassiri, and Dr. Vanhaesebroeck provided critical reviews of the manuscript. Dr. Oudit was the Corresponding author who involved with concept formation and supervised the project.

Dedications

This dissertation is dedicated to my beloved husband Yongzhe, my sister and brother, and my parents who have provided constant supports and encouragement during my graduate study.

Acknowledgements

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List of Abbreviations

4E-BP1	eIF-4E-binding protein 1
AC	Adenylyl cyclase
ACE	Angiotensin-converting enzyme
ACEI	ACE inhibitor
AKAP	A-kinase anchoring protein
AMPK	AMP-activated protein kinase
Ang I	Angiotensin I
ANP	Atrial natriuretic peptide
ARB	Ang II receptor blocker
ATP	Adenosine triphosphate
AT_1	Ang II receptor-1
β-MHC	β-myosin heavy chain
β-blocker	β-adrenergic receptor blocker
Bcl-2	B-cell lymphoma 2
BNP	Brain natriuretic peptide
BSA	Bovine serum albumin
CABG	Coronary artery bypass graft
CaMKII	Calmodulin-dependent protein kinase II
cDNA	Complementary DNA
CREB	cAMP-response element-binding protein
CVD	Cardiovascular disease
DAMP	Danger-associated molecular patterns
ECG	Electrocardiogram
ECM	Extracellular matrix
EF	Ejection fraction
EGF	Epidermal growth factor
eIF-4E	Eukaryotic initiation factor 4E
ERK	Extracellular signal-regulated kinase
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
ET_A	ET-1 receptor A
FAC	Fractional area change

FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FOXO	Forkhead Box O
GEF	Guanine nucleotide exchange factors
GLS	Global longitudinal strain
GLUT4	Glucose transporter type 4
GPCR	G protein-coupled receptor
GRK-2	G protein-coupled receptor kinase-2
GSK3β	Glycogen synthase kinase 3β
HCAEC	Human coronary artery endothelial cell
HDAC	Histone deacetylase
HELP	Human Explanted Heart Program
HER2	Human epidermal growth factor receptor 2
HFrEF	Heart failure with reduced ejection fraction
HIF1α	Hypoxia-inducible factor 1α
HOPE	Human Organ Procurement and Exchange program
HUVEC	Human umbilical vein endothelial cell
ICAM	Intercellular adhesion molecule
IGF-1	Insulin-like growth factor 1
IHD	Ischemic heart disease
IL	Interleukin
IMM	Inner mitochondrial membrane
IP3	Inositol phospholipids into inositol 1,4,5-trisphosphate
IP	Intraperitoneal
LAD	Left anterior descending artery
LVESV	Left ventricular end-systolic volume
LVEDV	Left ventricular end-diastolic volume
LTCC	L-type calcium channel
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential media
MI	Myocardial infarction
miRNA	microRNA
MMP	Matrix metalloproteinase
mPTP	Mitochondrial permeability transition pore
mRNA	messenger RNA

mTORC	Mammalian target of rapamycin complex
NFC	Non-failing control
NO	Nitric oxide
OCT	Optimal cutting temperature compound
OMM	Outer mitochondrial membrane
p110α-αMHC	Mice with cardiomyocyte specific PI3Kα inactivation
p110αFlx	Control littermates with floxed p110α alleles
p110αTie2	Mice with endothelial specific PI3Kα inactivation
p110β-αMHC	Mice with cardiomyocyte specific PI3Kβ inactivation
p110βFlx	Mice with endothelial specific PI3Kβ inactivation
p110βTie2	Control littermates with floxed p110β alleles
PCR	Polymerase chain reaction
PDE3	Phosphodiesterase 3
PDK1	Phosphoinositide-dependent kinase 1
PH	Pleckstrin homology
PI	Propidium iodide
PI(3,4,5)P3, PIP3	PtdIns (3,4,5)-trisphosphate
PI3K	Phosphoinositide 3-kinase
PI(4,5)P2, PIP2	PtdIns (4,5)-biphosphate
PKA	Protein kinase A
PKB, Akt	Protein kinase B
PKC	protein kinase C
PLB	Phospholamban
PLCβ	Phospholipase C β
PtdIns	Phosphatidylinositol
PTEN	Phosphatase and tensin homolog
RAS	Renin-angiotensin system
Rheb	Ras homologue enriched in brain
RIP	Receptor interacting protein
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RT-PCR	Real-time polymerase chain reaction
SERCA2a	Sarcoplasmic reticulum Ca^{2+}-ATPase 2a
SR	Sarcoplasmic reticulum
STAT3	Signal transducer and activator of transcription 3

S1P	Sphingosine-1-phosphate
S6K	S6 kinase
SDF1α	Stromal cell-derived factor 1-α
SGK	Glucocorticoid-regulated kinase
sip110β	Small interfering RNA against human p110β
SREBP1c	Sterol regulatory element-binding protein
s-siRNA	Scrambled small interfering RNA
SV	Stroke volume
TAK1	TGF-β-activated kinase
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitors of the metalloproteinase
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TRAIL	TNF-related apoptosis-inducing ligand
TSC	Tuberous sclerosis protein
TTC	Triphenyl Tetrazolium Chloride
TUNEL	DeadEnd Fluorometric Terminal Deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick-End Labeling
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
Vps34	Vacuolar protein sorting 34
WMSI	Wall motion score index
WT	Wildtype

Chapter 1

Introduction

1.1. Post-infarction Cardiac Remodeling

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide, which claimed 17.6 million deaths in the world in 2015 and 2016[1,2]. Ascribed to improved medical care and increased awareness of primary prevention, deaths attributable to CVD have declined slightly for the last two decades in advanced regions such as United States[3]. However, with the growth of the ageing population who are susceptible to CVD, CVD burden has increased constantly. Globally, deaths from CVD increased by 14.5% between 2006 and 2016[2]. And the total costs of CVD have been projected to reach 1.1 trillion in 2035 in United State[3]. Ischemic heart disease (IHD) has remained the dominant cause of CVD mortality in the world. In 2016, IHD claimed 9 million lives globally; and it is the leading CVD cause in United States between 1990 and 2016, making up 43.2% CVD in 2016[2-4]. Accounting for the majority of IHD morbidity and mortality, myocardial infarction (MI) is defined as the presentation of myocardial death resulted from myocardial ischemia which is usually due to the rupture or erosion of atherosclerotic coronary plaques[5,6]. MI has experienced a significant decline in incidence and mortality worldwide with variation among regions[7-10]. Nevertheless, it remains the leading contributor to the socioeconomic burden of disease, and it is commonly progressed to post-infarction heart failure with reduced ejection fraction (HFrEF) as the post-MI life expectancy increases[11,12]. Therefore, we should take MI seriously and advance our knowledge regarding the pathophysiologic basis and cardiac remodeling following MI, which might allow us to better identify potential therapeutic targets and to consequently improve the clinical outcomes in patients with MI, easing the burden of MI in the world.

1.1.1. Pathophysiology of Post-infarction Cardiac Remodeling

Following MI, the heart experiences a series of structural and functional changes, termed post-infarction cardiac remodeling, which is governed by cellular and molecular mechanisms and characterized by ventricular dilation, eccentric hypertrophy, and fibrotic scar formation[13]. These finely orchestrated modifications promote infarct healing and beneficial compensatory responses, maintaining cardiac structural integrity and cardiac pumping (Figure 1.1). However, the adaptive processes eventually become detrimental as the survived myocardium fails to compensate the increase in ventricular wall stress; and the infarcted heart develops adverse ventricular remodeling and progresses to HFrHF[12,14].
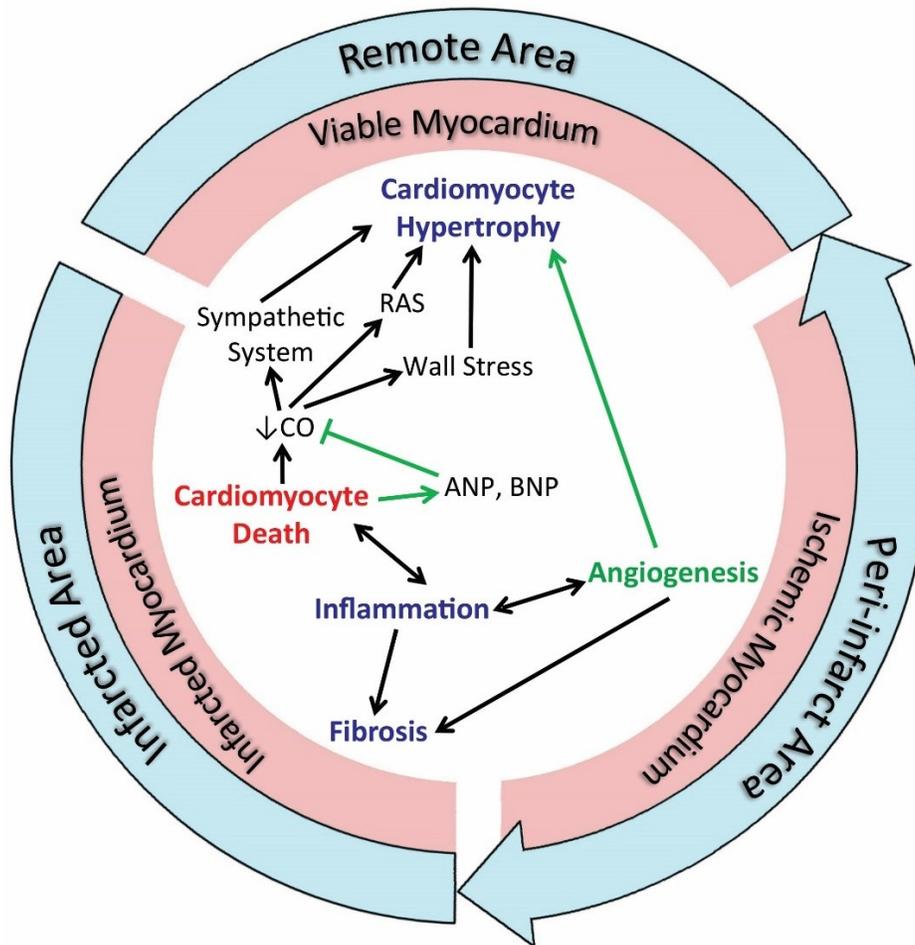
Figure 1. 1. Post-infarction cardiac remodeling is a finely orchestrated response. It involves in myocardium death, inflammation, cardiomyocyte hypertrophy, fibrosis, and angiogenesis, attempting to maintain cardiac structural integrity and cardiac function.

Even though cardiomyocytes in mammalian species are resistant to transient coronary ischemia, prolonged myocardial ischemia causes irreversible cardiomyocyte death through necrosis, apoptosis, and autophagic cell death[15], leading to detrimental outcomes as adult cardiomyocytes have minimum capacity to regenerate. Systemically, the loss of myocardium, which decreases cardiac function and causes substantial hemodynamic perturbation, leads to various detrimental actions in the cardiovascular system largely by the activation of sympathetic system and renin-angiotensin system (RAS). These systemic changes mediate cardiac hypertrophy, induce hypertensive responses, and exacerbate cardiac remodeling. Within the heart, the release of danger-associated molecular patterns (DAMPs, e.g., high mobility box 1 (HMGB1), mitochondrial DNA, and matricellular proteins) from necrotic cells, damaged extracellular matrix (ECM), and stressed cells stimulate the expression and the release of

inflammatory cytokines (e.g., tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6) and chemokines (e.g., monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein 2α (MIP-2α)) from survived cells through pattern-recognition molecules such as Toll-like receptors (TLRs), which leads to the recruitment of leukocytes, activation of vascular endothelia, and subsequent rolling and infiltration of the circulating leukocytes into the damaged myocardium[16]. The infiltration of neutrophils and monocytes further amplifies the inflammatory response through releasing more inflammatory mediators and induces the digestion of the damaged matrix and the clearance of dead cells and matrix debris. This proinflammatory response, with neutrophils, Ly6C^{high}CCR2^{high} monocytes, and M1 (classic) macrophages as the dominant players, paves the way for infarct healing and reparative remodeling. As the proinflammation resolves, anti-inflammatory pathways are activated, which are characterized by the accumulation of Ly6C^{low}CCR2^{low} monocytes and M2 (alternative) macrophages. Augmenting the expression and release of anti-inflammatory cytokines and growth factors (e.g., IL-10, transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF)), these inflammatory cells support collagen scar formation and angiogenesis through promoting the proliferation and differentiation of fibroblasts and endothelial cells. In addition to neutrophils, monocytes, and macrophages, dendritic cells and T-lymphocytes also contribute to inflammatory resolution and tissue repair. This timely and spatial regulation of post-MI pro-inflammatory and anti-inflammatory responses is required for proper infarct repair, but an inadequate, prolonged, or remote myocardium-localized activation of inflammation could result in detrimental effects on the heart including the induction of cardiomyocyte death, excessive reactive oxygen species production, and unwanted fibrosis[17].

In response to the increased fibrogenic mediators, especially cytokine TGF-β, the best described regulator of cardiac fibrosis, fibroblasts differentiate into myofibroblasts which express contractile smooth muscle actin and contribute to the synthesis and deposition of type I and type III collagen, forming a fibrotic scar to replace damaged myocardium. Besides heavily involved in fibrotic responses, cardiac myofibroblasts have been reported to participate in the engulfment of apoptotic cells in the infarcted heart, contributing to infarct size limitation through obtaining anti-inflammatory properties upon engulfment[18]. As the fibrogenic signals withdraw, the infarct repair goes into the maturation phase, characterized by the cross-linked matrix proteins and the deactivation of myofibroblasts. While adequate collagen scar formation is critical to maintain the integrity of the infarcted heart, the presence of fibrosis in the non-infarct area could further compromise ventricular compliance, affecting the function of the heart. Overall, inflammation

and the replacement of dead tissue by fibrotic scar are critical infarct healing processes that maintain the structural integrity of the heart.

Besides the key responses mentioned above, many processes take place to support cardiac remodeling and to improve cardiac function. The survived cardiomyocytes undergo hypertrophy to counteract the loss of cardiomyocytes and the increase in wall stresses. This hypertrophic response is stimulated by the perturbations of circulatory hemodynamics which activate the sympathetic nervous system, the systemic and local RAS, and natriuretic peptide (atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP)) release. Moreover, the infarct healing process is accompanied by an intense angiogenic response which amplifies post-MI revascularization to salvage ischemic cardiomyocytes, to support the healing process and cardiomyocyte hypertrophy with adequate oxygen and nutrients, and to attenuate the HF transition[19-21]. A high plasma VEGF level in MI patients has been associated with better prognosis[22]. In addition, during injury repair, the cardiac interstitial tissue constantly undergoes remodeling, predominantly regulated by the balance between matrix metalloproteinases (MMPs) and tissue inhibitors of the metalloproteinase (TIMPs). The initial degradation of the ECM by serine proteases and MMPs, on one hand, results in infarct expansion and ventricular dilation, leading to the elevation of wall stresses. On the other hand, it is accompanied by the creation of a provisional matrix which serves as a scaffold to mediate cardiac repair, including inflammation, angiogenesis, and collagen scar formation. Subsequently, the ECM continues being degraded and deposited until the stabilization of the distending stress. Additionally, cardiac remodeling is associated with the metabolic switch which the heart shifts from relying on mitochondrial fatty acid oxidation-produced adenosine triphosphate (ATP) to increasing the usage of glycolysis-contributed ATP production[23]. This change compromises myocardial energy production, contributing to impaired cardiac contractility and the transition to HF. Furthermore, electrical remodeling following MI, altered electrical properties of the heart, affects the outcomes of patients with an ischemic heart by compromising cardiac functions and potentially triggering cardiac arrhythmia[24]. Last but not least, cardiac remodeling also causes systemic alterations triggering responses in distant organs such as liver and spleen, which is associated with various cell mobilization and active molecule secretion during remodeling processes, affecting gene expression and function of other organs[25-27].

It is essential to recognize that various cell types participate in orchestrating the complex and integral post-infarct responses, including cardiomyocytes and noncardiomyocytes. Effective post-infarct cardiac repair and cardiac function depend on extensive intercellular and cell-ECM

communication in the heart in a timely, coordinated manner. There is no doubt that there are still knowledge gaps on the roles of these cell types and their contribution to cardiac remodeling. Among the integrated cellular players in a healthy heart, cardiomyocytes occupy about 70% of the heart volume but only constitute approximately 30% of the cells in a mammalian adult heart, whereas endothelial cells have recently been identified as the most abundant cell population, made up more than 50% of all cells in the heart[28,29]. Moreover, cardiomyocytes and cardiac vasculature constantly interplay with each other to achieve a dynamic and homeostatic state. Whilst cardiomyocytes affect the vascular tone and vascular growth through paracrine signals such as VEGFs, the cardiac vasculature supports cardiomyocyte survival and growth, regulating the heart size and cardiac function[30]. In addition, cardiomyocytes and endothelial cells, important components in post-infarct cardiac remodeling, are promising targets in mitigating ischemic injury. A considerable amount of research targeting the inflammatory and fibrotic responses in ischemic injury has shown conflicting results because of the requirement of a delicate balance among the pleiotropic, cell-specific players in cardiac repair. Thus, in this dissertation, I focus on the potential of targeting cardiomyocytes and endothelial cells in ischemic heart treatments. The current understanding of the role of cardiomyocytes and endothelial cells in post-infarction cardiac remodeling is summarized below.

1.1.2. Cardiomyocytes: Essential in Cardiac Function Regulation

Cardiac remodeling following coronary artery occlusion has profound effects on cardiomyocytes in both the affected and unaffected areas. Cardiomyocytes, especially those in the infarcted region, struggle to survive, while the survived cardiomyocytes undergo hypertrophy attempting to maintain cardiac function. Cardiomyocyte death and hypertrophy are the two main results responding to stress as an adult heart has negligible regenerative capacity.

1.1.2.1. Cardiomyocyte Death

The abruption of the coronary blood flow triggers the loss of cardiomyocytes extending from the subendocardium to the subepicardium[31,32], which initiates the post-infarct cardiac remodeling; and the magnitude of initial myocardial loss controls the severity of remodeling. Even though timely coronary artery patency is achieved, a paradoxical exacerbation of cardiomyocyte death might occur during the restoration of blood flow and reoxygenation, termed ischemia-reperfusion (IR) injury. IR injury is related to increased reactive oxygen species (ROS) production such as peroxynitrite, elevated inflammation, and increased vascular impedance[33]. In addition, the cardiomyocytes resident in the remote area, which survival through the acute event, could also undergo cell death because of the disruption of pro-survival and pro-death

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signals during chronic post-infarct cardiac remodeling. Nonetheless, both hypoxia-mediated and reoxygenation-induced injury cause cardiomyocyte death through apoptosis, necrosis, and autophagy-associated cell death[33,34]. A high magnitude of both necrosis and apoptosis are implicated in the cardiomyocyte death in the infarcted region and in the development of HF.

Necrosis, the predominant form of cell death in the infarcted heart, is characterized by cell swelling and the subsequent rupture of the cell membrane, eliciting intensely inflammatory responses because of the release of intracellular contents. It is now recognized that this form of cell death can be regulated by signaling events including receptor interacting protein 3 (RIP3)-dependent necroptosis and mitochondrial permeability transition pore (mPTP) regulated necrosis. During MI, necroptosis is triggered by the activation of death receptor pathway by death receptor ligands such as TNF-α which initiate the downstream assembly of protein complexes, leading to RIP1-RIP3 interaction and subsequent necroptotic cell death when caspase-8 is inhibited[35]. Moreover, calcium overloading condition leads to the opening of mPTP in the inner mitochondrial membrane (IMM). The resulting destruction of the electrochemical proton gradient across IMM causes mitochondrial swelling and rupture.

Apoptosis, also termed programmed cell death, involves the shrinkage and fragmentation of the cells, producing plasma membrane-enclosed apoptotic bodies. Based on the mechanistic differences, it can be separated into mitochondrion-involving intrinsic pathways and cell surface death receptor-mediated extrinsic pathways[15]. They are both critically implicated in cardiomyocyte death in the infarcted heart. The intrinsic pathway is regulated by the interactions among the B-cell lymphoma 2 (Bcl-2) family members, controlling the permeabilization of the outer mitochondrial membrane (OMM). Under MI, the increased cell stress disrupts the balance between BH3-only proapoptotic Bcl-2 family proteins (e.g., Bad, Bid, and Puma) and antiapoptotic proteins (e.g., Bcl-2, Bcl-Xl, and Mcl-1), leading to the activation and pore formation of OMM-located multidomain proapoptotic Bcl-2 family members Bax and Bak. This results in the escape of mitochondrial inter-membranous space contents such as cytochrome c and Smac/DIABLO and subsequent formation of the apoptosome complex and caspase activation. The extrinsic apoptotic pathway is initiated through death receptors[36]. Triggering by FAS ligand, TNF-α, or TNF-related apoptosis-inducing ligand (TRAIL), death receptors recruit downstream adaptors to form protein complexes which cause the activation of caspase cascades, leading to apoptotic cell death.

Autophagy is a housekeeping and pro-survival mechanism that recycle dysfunctional or unnecessary components to replenish energy. During autophagy, membrane besieged

intracellular organelles and parts of the cytoplasm, termed autophagosomes, are delivered to lysosomes for degradation. Research has shown that the enhancement of autophagy under myocardial ischemia through AMP-activated protein kinase (AMPK) activation protects cardiomyocytes from cell death[37,38]. However, it can also mediate cellular demise through apoptotic and autophagic killing[39,40]. Autophagic cell death has been described as massive vacuolization and ubiquitin accumulations, which might be induced by excessive degradation of cellular components[34,41]. Further investigations are required to illustrate autophagic effects on cardiomyocyte survival and death.

Cardiomyocyte survival and death through necrosis, apoptosis, or autophagy are governed by intricate signaling pathways and multiple effector molecules which are interplaying with and influencing each other. For instance, death receptors have been shown to trigger survival, apoptosis, and necroptosis signaling depending on the intracellular conditions. And mitochondria can be the center of both apoptotic and necroptotic cell death, whilst mitochondrion-trigger necroptosis also induces apoptosis when mitochondrial breakdown releases mitochondrial contents. Moreover, Bcl-2 family proteins regulate both apoptosis and autophagy. Nevertheless, how these processes interplay and balance in the MI condition to control cardiomyocyte survival and death is not fully understood. The upcoming challenges to uncover these complex relationships should be taken to guild context-specific pharmacological therapies.

1.1.2.2. Cardiomyocyte Hypertrophy

To maintain adequate contractile function and counterbalance the elevated ventricular wall stress, the remained cardiomyocytes primarily undergo hypertrophy which is characterized by the increase in protein synthesis and cell volume, especially contributed to the increased series replication of sarcomeres. This change initially is beneficial by compensating the cardiac functional loss; however, this adaptive hypertrophy eventually might become detrimental as it fails to normalize the increased wall stress and to control chamber dilation, generally termed eccentric hypertrophy. Thus, this pathological cardiac hypertrophy is detrimental and associated with increased morbidity and mortality when the pathological condition remains[42,43]. Distinct from physiological cardiac hypertrophy which displays normal cardiac morphology and function during postnatal cardiac growth and pregnancy- or exercise-induced cardiac hypertrophy, the pathological hypertrophy induced by MI is associated with cell death, cardiac metabolism alteration, cardiac fibrosis, and contractile dysfunction. In the infarcted heart, cardiac hypertrophy is stimulated by a variety of stimuli, including neurohormonal factors, myocardial

stretch, and other paracrine/autocrine stimuli. Through binding to membrane receptors, these factors activate multiple intracellular signaling cascades which subsequently alter cardiac gene profiles, resulting in increased cardiac contractile proteins and sarcomere assembly. This is characterized by upregulated β-myosin heavy chain (β-MHC) and downregulated α-MHC, increased fetal gene programming (e.g., ANP and BNP), and decreased calcium-handling proteins (e.g., sarcoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a)), struggling to achieve the more economical contractile function[44,45].

The major trigger of pathological hypertrophy is neurohumoral factors elicited by MI-triggered mechanical stress. The catecholamine (e.g., norepinephrine and epinephrine) released from sympathetic nerve terminals and the adrenal medulla activates adrenergic receptors on cardiomyocytes, triggering the hypertrophic effects. Meanwhile, compromised cardiac function and activated sympathetic nervous system trigger the release of renin from the renal juxtaglomerular apparatus, activating RAAS. Produced and released from the liver, angiotensinogen is cleaved by renin to form angiotensin I (Ang I) which further transforms to Ang II by angiotensin-converting enzyme (ACE) primarily located in the pulmonary vasculature. Besides the increase in the systemic RAS, the cardiac Ang II production also increases in response to the upregulated angiotensinogen gene expression and the increased local ACE activity. The increased Ang II stimulates hypertrophic signaling by binding to the Ang II receptor-1 (AT_1) on the cardiomyocytes[46]. The enhanced Ang II activity enhances norepinephrine production, and vice versa. Moreover, Ang II and norepinephrine enhance the release of endothelin-1 (ET-1) from the cardiovascular system. Then, ET-1 triggers cardiomyocyte hypertrophy through binding ET-1 receptor A (ET_A)[47]. Belonging to G protein-coupled receptor (GPCR) family, adrenergic receptors, AT_1, and ET_A activation initiate the multiple intracellular signaling pathways to induce cardiac hypertrophy[48]. The activation GPCRs causes the dissociation of the Gα and Gβγ subunits. While the former subsequently mediates inotropy and hypertrophic effects through Gαs-induced, cyclic adenosine monophosphate (cAMP)-dependent or Gαq-directed, phospholipase C β (PLCβ)-mediated mechanisms, the latter subunit triggers phosphoinositide 3-kinase (PI3K)/Akt cascades to regulate cardiac hypertrophy[49]. Adenylyl cyclase (AC)-mediated generation of cAMP activates protein kinase A (PKA) which subsequently phosphorylates contractility-regulated substrates such as ryanodine receptor, phospholamban (PLB), and L-type calcium channel (LTCC) to enhance cardiac contractility with the assistance of scaffolding protein-A-kinase anchoring proteins (AKAPs). And AKAPs also mediate hypertrophic gene transcription through several mechanisms. For instance, the activation of AKAP cargo proteins calcium-sensitive calcineurin and phosphodiesterase regulate

transcription through transcription factor NFAT and MAPK signaling respectively[50,51]. Another pro-hypertrophic player downstream GPCR signaling, PLCβ, converts membrane inositol phospholipids into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. While IP3 mobilizes sarcoplasmic reticulum (SR) calcium through IP3 receptor binding, diacylglycerol-mediated protein kinase C (PKC) and ensuing PKD activation play critical roles in cardiac hypertrophy by histone deacetylase (HDAC) inactivation and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway activation[52-54]. Furthermore, calcium transients regulate cardiomyocyte growth and hypertrophic gene programming through the activation of calcium-sensitive calcineurin and calmodulin-dependent protein kinase II (CaMKII)[55-57]. Finally, GPCR activation also triggers cAMP-dependent activation of the small GTPase Rac and Ras which in turn activate downstream cardiac hypertrophic cascades[58,59].

In addition to the contribution of neurohormonal responses in cardiac hypertrophy, mechanical stretch can directly regulate transcriptional profiles to promote hypertrophic growth of cardiomyocytes[60]. Mechanotransduction of stretch-induced hypertrophy is mediated via stretch-sensing proteins that convert extracellular signals to intracellular information. Among them, integrins are the center of myocardial stretch-induced hypertrophic signaling by linking ECM with contractile sarcomeres and triggering intracellular signaling pathways such as PI3K/Akt and ERK1/2 pathways[61,62].

Finally, paracrine and autocrine signaling from IL-6[63], insulin-like growth factor 1 (IGF-1)[64], and TGF-β[65] is implicated in stress-induced cardiac hypertrophy. These ligands promote hypertrophic growth through activation of downstream effectors including signal transducer and activator of transcription 3 (STAT3)[66], PI3K/Akt, and TGF-β-activated kinase (TAK1)/MAPK[65,67]. To balance hypertrophic growth, the stimulation of Ang II and ET-1 and mechanical strain induce the production of ANP and BNP which negatively modulate cardiac hypertrophy through atrial natriuretic peptide receptors (NPR)/guanylyl cyclase/cyclic GMP (cGMP) signaling[68].

Thus, during post-infarct cardiac remodeling, the appropriateness of cardiac hypertrophy is of great importance and clinically relevant. And these hypertrophic responses are highly coordinated with a complex crosstalk within intracellular signaling pathways and stimuli.

1.1.3. Cardiac Endothelial Cells: A Modulatory Component of Cardiac Function

Cardiac endothelial cells and the vasculature are key regulators in cardiac health, which guarantees the adequate supply of oxygen and nutrients, supports cardiac growth, regulates vascular tone, prevents thrombotic formation, and maintains myocardial homeostasis through

paracrine signals such as apelin. With these key functions, endothelial cells participate in the pathogenesis of heart diseases such as atherosclerosis, myocardial infarction, and cardiomyopathy. Here, I summarize the roles of endothelial cells in post-infarct cardiac remodeling.

Although mostly remaining quiescent, the endothelium can be rapidly activated in response to myocardial injury, orchestrating the processes in cardiac remodeling. First, the activation of endothelial cells at the onset of MI is required for the inflammatory responses as they facilitate the recruitment and infiltration of immune cells. Responding to the increased pro-inflammatory signals, endothelial cells are activated with the upregulation and presence of adhesion molecules such as P-selectin, E-selectin, and intercellular adhesion molecule (ICAM) on the lumen surface of the plasma membrane, which promotes the binding and transendothelial migration of circulating leukocytes. Moreover, the activation of the endothelium increases vascular permeability as the intercellular junctions between endothelial cells become compromised, facilitating the inflammatory responses. In addition, the balance between vasodilation and vasoconstriction after MI is altered, which is associated with altered levels of vascular tone regulators and different responses of coronary artery toward these regulators. Endothelial nitric oxide synthase (eNOS) level have been demonstrated to be increased in the infarct and peri-infarct areas after MI, and vasodilator nitric oxide (NO) generated by eNOS has displayed cardioprotective effects with improved endothelial vasomotor dysfunction[69,70]. The coronary vasoconstriction effects from vasoconstrictors such as Ang II and endothelin are reduced, while the vasodilator influence of NO is reduced as well[71,72].

Most importantly, endothelial cells can undergo angiogenesis, the construction of new microvessels from existing capillaries, to support inflammatory and fibrotic changes and to keep pace with cardiomyocyte hypertrophy after MI, attenuating the progressive transition to HF. And this is a major form of damaged vessel reconstruction in the ischemic heart. Multiple growth factor families, cytokines, and chemokine have angiogenic effects and contribute to angiogenic processes, including VEGF, IGF, IL-8, and stromal cell-derived factor 1-α (SDF1α). After MI, the myocardium upregulates angiogenic signals through several pathways. Firstly, the ischemic myocardium increases the expression of angiogenic factors through hypoxic stabilization of hypoxia-inducible factor 1α (HIF1α) and subsequently enhancing the HIF1 transcriptional activity, including VEGF and NOS transcription[73,74]. Secondly, anti-inflammatory cells present during cardiac repair processes, such as reparative macrophage phenotype and fibroblast, also synthesize and release angiogenic factors[75,76]. Finally, mechanical stresses have been

demonstrated to increase angiogenic factor expression which might be associated with HIF1α activity[77]. Among all these angiogenic signals, VEGF is a key and well-studied one that has shown to be significantly upregulated after acute MI; and all these signals are tightly coordinated to guarantee functional vasculature formation[78-80]. Activated by angiogenic signals, endothelial cells detach from the basement membrane and migrate into the matrix, which is facilitated by the increased vascular permeability and the ECM remodeling. Sensing angiogenic signals, a specialized endothelial cell-tip cell is selected and migrates toward the higher gradient of angiogenic signals, while the neighboring endothelial cells-stalk cells proliferate to elongate the sprouts. Eventually, a vessel lumen is formed, and the newly formed vessels are stabilized by mural cell recruitment and basement membrane reestablishment.

Besides angiogenesis, vessels can grow in other ways after MI, including vasculogenesis, arteriogenesis, and collateral growth. Unlike angiogenesis, vasculogenesis forms microvessels by mobilizing endothelial progenitor cells to the ischemic area and incorporating themselves into existing vessels[81,82]. Arteriogenesis refers to the vessel maturation by mural cells and vessel enlargement, while collateral growth highlights the expansion of pre-existing vessels. These methods coordinate with each other to achieve a more extensive vascular network. The paracrine action of mobilization of stem and progenitor cells enhances angiogenesis with the secretion of angiogenic factors such as VEGF[83]. Arteriogenesis and collateral growth are part of the new vessel maturation and remodeling. Via these processes, the increase in vascular density has the potential to salvage ischemic myocardium and to prevent the transition to HF. Among all these methods, capillary angiogenesis is a promising therapeutic target to help manage MI. As a highly vascularized organ, the heart contains many endothelial cells which provide angiogenic potential in an ischemic event. In the infarct area, angiogenesis facilitates inflammatory responses and scar formation. Capillary formation in the border zone distributes blood flow to the ischemic but viable myocardium. And the newly formed capillaries in the remote area support long-term cardiac remodeling and cardiac function.

1.1.4. Novel Therapeutic Intervention

Introduction of timely coronary reperfusion through thrombolytic therapy, percutaneous coronary intervention, and/or coronary artery bypass graft (CABG) surgery has significantly improved the prognosis of acute MI patients, which salvages ischemic myocardium and subsequently attenuates post-infarction cardiac remodeling. Additionally, pharmacological interventions have consistently improved patient outcomes. ACE inhibitors (ACEIs), Ang II

receptor blockers (ARBs), and β-adrenergic receptor blockers (β-blockers) have improved survival rates and attenuated cardiac remodeling despite that the detailed mechanisms underlying these drugs remain unclear[14]. Partially, these drugs have contributed to the regulation of cardiomyocyte hypertrophy, cell death, and fibrosis, eventually ameliorating the transition to adverse ventricular remodeling. Moreover, combined therapies with antiplatelet, anticoagulation, and lipid-lowering agents have become an essential component in the treatment of MI, which prevents the reoccurrence of a new thrombotic event. Unfortunately, despite the widespread use of these interventions in the MI population, the mortality and morbidity of MI and MI-related HF remain high. Thus, novel therapeutic strategies are required to prevent or attenuate post-infarction adverse remodeling.

Beside early revascularization, other therapeutics preventing programmed cell death have been investigated, attempting to further improve the clinical outcomes of MI patients. Regardless of the beneficial effect of TNF-α inhibition in animal MI models, clinical trials of anti-TNF therapy in HF patients were disappointing, raising the necessity of a better understanding of TNF-α in HF[84,85]. Cyclosporine, an immunosuppressant with inhibitory effects on mPTP opening, has demonstrated no cardioprotective effect on acute MI patients on survival, cardiac function, and rehospitalization[86]. Although these therapeutic strategies hold limited promise in MI treatment, cell death inhibitors still have the potential to be cardioprotective agents.

Several strategies to enhance vascularization in the damaged heart have been proposed and tested in clinical trials. Because of the role of growth factor or cytokine-mediated vascular growth, growth factors and cytokines have potential therapeutic effects in MI via enhancing vascularization. Gene therapy targeting growth factors, such as VEGF, has resulted in potential benefit despite the statistical underpower[87,88]. Cell therapy, transplanting bone marrow-derived stem cells, endothelial progenitor cells, or other pluripotent cells, has been tested based on the notion that pluripotent stem cells have the capability of differentiating into different cell types which potentially repairs or regenerate the damaged myocardium, especially contributing to vascular repair and neovascularization. Despite that current clinical trials have shown inconclusive results and low statistical power, several have demonstrated cardioprotective effects of cell therapy in MI patients by improving cardiac function[89,90].

In conclusion, translating encouraging preclinical data to clinical trials is challenging and current findings of these approaches in the clinical context have yielded mixed results. But these novel therapies have remained promising targets in tackling MI. The therapeutic window of these methods should be examined further as it may be very narrow for some therapies. And a

combination of therapies might be needed to elevate clinical benefits. Moreover, better elucidation of the complex mechanisms of post-infarct cardiac remodeling has continued to yield significant benefit by providing promising therapeutic targets. The clarification of the effects of signaling pathways and cellular targets is likely to provide essential information in the promisingly multidisciplinary treatment because targeting a single pathway or an individual cell type is inefficient due to these intricate and overlapping targets.

1.2. The PI3K Isoforms and Signaling

Phosphoinositide 3-kinases are a family of conserved lipid kinases that phosphorylate the third hydroxyl group of the inositol ring of phosphatidylinositol (PtdIns), which in turn regulate an extraordinarily wide range of cellular functions involving nutrient sensing, growth and metabolism control, and aging. They are categorized into three major classes (Class I, II, and III) based on structural and functional specificity. Class II PI3Ks are monomers with a carboxy-terminal C2 domain, including PI3K-C2α, PI3K-C2β, and PI3K-C2γ, which specifically phosphorylate PtdIns and PtdIns(4)P. Class III PI3K only has one member-vacuolar protein sorting 34 (Vps34), a heterodimeric complex, which prefers targeting PtdIns and critically involves in vesicle trafficking and autophagic processes. In the dissertation, I will focus on the Class I PI3Ks (which will be simplified as PI3Ks at the rest of the dissertation), their signaling networks, and how they function in the cardiovascular system.

Class I PI3Ks are of the most interesting of all classes of PI3Ks because they are vital in modulating cellular regulatory processes, including cell survival, cell growth, cell cycle[91,92], glucose metabolism[93], and actin dynamics and cell motility. They are heterodimers which consist of a p110 catalytic subunit (p110α, β, δ, and γ) and a regulatory subunit, converting PtdIns (4,5)-biphosphate (PI(4,5)P2, PIP2) to PtdIns (3,4,5)-trisphosphate (PI(3,4,5)P3, PIP3). Coupling with different types of regulatory subunit, Class I PI3Ks are further divided into two subclasses, Class I$_A$ and I$_B$. Three Class I$_A$ PI3Ks, PI3Kα, PI3Kβ, and PI3Kδ, are encoded by *pik3ca* (3q26.32), *pik3cb* (3q22.3), and *pik3cd* (1p36.22) gene specifically. They couple with a p85 family regulatory subunit (p85α, p55α, p50α, p85β, and p55γ), predominantly activated by receptor tyrosine kinases (RTKs) such as insulin receptor, IGF-1 receptor, and VEGF receptor (VEGFR) via the regulatory subunit-activated RTK interaction. Besides activated by RTKs, PI3Kβ has been shown to regulate GPCRs-dependent signaling through G$_{βγ}$ heterodimer-p110β binding[94]. Despite that both PI3Kα and PI3Kβ are ubiquitously expressed, PI3Kα is the dominant isoform responding to RTK activation in many cell types[95], whereas PI3Kδ is highly restricted to leukocytes and involves in immune responses. Meanwhile, the only Class I$_B$ PI3K-

PI3Kγ, encoded by *pik3cg* (7q22.3) gene, is binding to a p101 or a p87 subunit, mediated by free $G_{\beta\gamma}$ subunits following the activation of GPCRs such as β adrenergic receptors, AT1, and ET_A. Besides activated by RTKs and/or GPCRs, PI3Ks can interact and be activated by Ras and Rho family small GTPases[96]. PI3Kα and PI3Kγ can also be activated by RAS, whereas PI3Kβ is stimulated by RAC1 and CDC42 downstream of GPCRs[97-99].

These various PI3K isoforms have distinct and redundant functions which are precisely balanced to maintain proper development. Studies on germline manipulation of specific PI3K isoforms have revealed that both PI3Kα and PI3Kβ are required for embryonic development as either deficiency of PI3Kα or PI3Kβ leads to early embryonic lethality due to severe proliferative defects or growth arrest at the blastocyst stage specifically[100-102], while deficiency of PI3Kγ or PI3Kδ is viable with impaired inflammatory responses[103,104]. However, mice with kinase-dead PI3Kβ develop normally, suggesting the importance of the kinase-independent function of PI3Kβ in prenatal development[105]. Despite that both PI3Kα and PI3Kβ bind to activated RTKs, PI3Kα is the main isoform transducing the RTK/Akt signaling such as the hepatic insulin signaling, while PI3Kβ catalytic activity is required to sustain long-term insulin signaling[105,106]. Moreover, PI3Kβ has been suggested to regulate basal Akt signaling because it is less inhibited by regulatory subunit binding than PI3Kα, proposing the function of PI3Kβ in the control of basal PI3K/Akt activity[107]. Research has also suggested a competitive role between them in which the less active PI3Kβ competes for receptor binding with the more active PI3Kα, which highlights the importance of PI3Kα and PI3Kβ in fine-tuning RTK-induced PI3K signaling[108]. Besides, PI3Kβ has been found in the nucleus where it regulates DNA replication, double-strand break DNA repair, and cell survival[109-111]. In addition, even though all PI3K isoforms are implicated in oncogenesis, PI3Kα is predominantly associated with the development and progression of solid tumors such as breast, cervical, lung, and head and neck cancer, whereas PI3Kβ majorly participates in prostate tumorigenesis driven by PTEN loss probably via transducing GPCR signaling[112-114]. Heavily present in hematopoietic cells, PI3Kδ and PI3Kγ are involved in hematopoietic and lymphoid malignancies, being promising targets in the management of these malignancies[115,116]. Because of that, selective inhibitors of PI3Kδ such as idelalisib and duvelisib have shown beneficial effects in chronic lymphocytic leukemia. Furthermore, PI3Kβ and PI3Kγ have been demonstrated with a kinase-independent, scaffolding roles. Whilst the former has participated in clatharin-mediated endocytosis and nuclear lamina organization[117,118], the latter has regulated phosphodiesterase 3 (PDE3)-mediated cAMP destruction[119]. In addition, highly expressed in hematopoietic cells, PI3Kδ and PI3Kγ exert biological functions relevant to

autoimmune disease, inflammation, and thrombosis[120]. Clearly, the PI3K isoforms display overlapped and distinct functions to coordinate complex intracellular signaling.

The action of Class I PI3Ks is antagonized by a tumor suppressor-phosphatase and tensin homolog (PTEN) which dephosphorylates PIP3 to PIP2. PIP3, the second messenger, in turn binds to pleckstrin homology (PH) domain containing effectors such as phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB/Akt), recruiting them to the plasma membrane where PDK1 phosphorylates Akt at Thr308. Maximal activation of Akt requires phosphorylation at both Thr308 and Ser473. The latter has been demonstrated to be phosphorylated by the mammalian target of rapamycin complex 2 (mTORC2); however, the regulation of this process is still under investigation and appears to be influenced by multiple factors. The activation of Akt results in phosphorylation of a wide spectrum of downstream substrates, controlling cell survival, proliferation, growth, migration, and others[121]; and the list of Akt substrates is expanding.

Akt is a key PI3K effector in the regulation of cell functions. First of all, Akt exerts pro-survival functions through many signaling pathway[122]. On one hand, it directly phosphorylates Bad to prevent activating the apoptotic signaling[123]; on the other hand, Akt enhances the pro-survival and anti-apoptotic gene expression through regulating transcription factors such as cAMP-response element-binding protein (CREB), NFκB, and Forkhead Box O (FOXOs)[124-126]. Secondly, Akt promotes cell growth by stimulating mTORC1-dependent protein synthesis and inhibiting protein degradation[127]. Akt inactivates tuberous sclerosis protein 2 (TSC2), reducing the GTPase-activating protein (GAP) activity of TSC1-TSC2 complex towards the small G-protein Ras homologue enriched in brain (Rheb), which triggers mTORC1 activation[128]. Then mTORC1 enhances protein synthesis through inhibition of eukaryotic initiation factor 4E (eIF-4E)-binding protein 1 (4E-BP1) and activation of S6 kinase (S6K). Moreover, Akt also relays the growth signals through inhibiting FOXOs and glycogen synthase kinase 3β (GSK3β) transcription factor[129,130]. Furthermore, Akt mediates cell cycle and proliferation by regulating mTORC1, FOXO, Mdm2, p21, p27, and Myc. Akt-dependent mTORC1 activation and FoxO inhibition enhance cell proliferation, which is related to Myc-mediated pro-proliferation activity[131-133]. Phosphorylation of Mdm2 by Akt elevates the degradation of the p53 tumor suppressor, which is associated with increased Mdm2 nuclear localization and its activity[134,135]. And p21 and p27 nuclear extrusion is promoted by Akt, which subsequently releases its cell proliferation-inhibiting activity[136,137]. In addition, Akt plays an important role in metabolism by promoting glucose uptake, glycogen synthesis, and lipogenesis. It enhances AS160-dependent membrane

translocation of glucose transporter type 4 (GLUT4) and promotes GLUT1 activity and recycling, speeding up GLUT4-mediated glucose uptake[138-140]. And Akt promotes glycogen synthesis by suppressing GSK3-regulated glycogen synthase inhibition, whilst it activates the transcription factor sterol regulatory element-binding protein (SREBP1c) to enhance hepatic lipogenesis via mTORC1 stimulation and INSIG2a suppression[141,142]. Last but not least, besides having substrates resident in the nucleus, Akt has been present in the nuclear compartment where it exerts multiple functions such as cell cycle, survival, and DNA repair[143]. Despite that the detailed processes of Akt nuclear translocation have not been fully unveiled, Akt has been demonstrated to translocate to the nuclei partially through association with other proteins[144]. Within the nuclei, Akt not only targets the aforementioned substrates such as FOXO, p21, and p27, it also interacts with other nuclear proteins such as Ebp1 and B23 to enhance cell survival[145-147]. Overall, Akt signaling network regulates a wide range of cellular functions, and a more comprehensive understanding of this network will provide key information in biomedical sciences.

Beyond Akt, PI3Ks/PDK1 also activates serum and glucocorticoid-regulated kinase (SGK) which processes structural similarity with the catalytic domain of Akt[148]. SGK1 has been demonstrated to share several downstream substrates with Akt such as p70s6 kinase and GSK3β, regulating cell size, cell survival, and protein synthesis[149]. And it also regulates the activity of late sodium current in the heart. Mice with cardiac SGK1 overexpression have developed mildly decreased ejection fraction at baseline and a higher lethal ventricular arrhythmia incidence after IR injury because of the positive effects of GSK1 on late sodium current, while dominant negative SGK1 expression has generated cardioprotective effect after transverse aortic constriction[150]. Moreover, PI3Ks also play a crucial role in cytoskeleton reorganization through PIP3-dependent, subcellular localization of guanine nucleotide exchange factors (GEFs) which consequently activate Rac proteins[93,151]. In addition, PI3K-driven PIP3 negatively regulate gelsolin activity to preserve actin cytoskeleton in the cardiomyocytes[152]. However, further investigations on the role of PI3K signaling beyond Akt are required.

Taken together, PI3K signaling, especially the PI3K/Akt pathway, plays a central role in cellular function, mediating critical cellular processes such as cell survival, growth, proliferation, and more (Figure 1.2). Consequently, it has been revealed to be critically involved in a wide range of human disorders, making this pathway a promising therapeutic target.
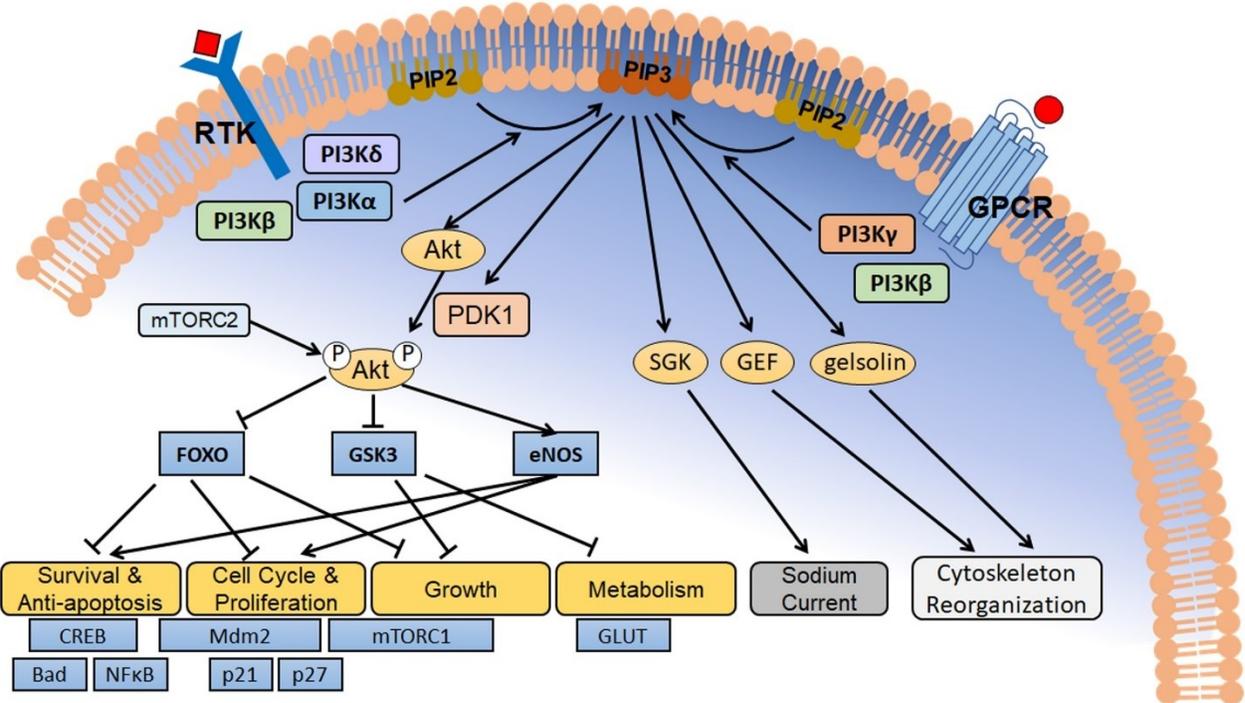
Figure 1. 2. PI3K signaling critically regulates cell survival, proliferation, cell growth, metabolism, and others.

1.2.1. PI3K/Akt Signaling in the Heart

Aberrations in PI3K signaling contribute to a wide range of human diseases, including cancer, diabetes, immunological disorders, overgrowth syndromes, and cardiovascular disease[121,153]. Here, I am going to highlight the roles of PI3K signaling in cardiac development and heart diseases. Firstly, PI3K signaling is important in cardiac postnatal growth. Suppression of PI3K signaling by loss of PDK1, deficiency of PI3K regulatory subunit, or enhanced GSK3β activity has led to a smaller heart and restriction of cardiac hypertrophy, whilst overexpression of this pathway through deleting PTEN gene or overexpression PI3K/Akt has generated the opposite effects[154-156]. Shiojima et al demonstrated that Akt expression participates in both physiological and pathological hypertrophy depending on the duration of Akt activation[21]. Secondly, PI3K pathway regulates cardiomyocyte viability under pathological conditions. PI3K/Akt activation has prevented cultured cardiomyocytes from oxidative stress-induced apoptosis and doxorubicin-induced cell death[157,158]. Cardiac-specific overexpression of Akt has improved cardiac function on a pressure overload-induced HF model by decreasing apoptosis and supporting angiogenesis[159]. Thirdly, PI3K/Akt signaling critically involves in the regulation of cardiac contractility. Mice with cardiac-specific Akt overexpression have shown increased

cardiac contractility[130]. PI3K/Akt signaling sustains calcium current, which is partially due to PI3K/Akt-dependent stabilization of L-type Ca^{2+} channels increasing its density at the plasma membrane[160,161]. However, the lack of PTEN has resulted in contractile dysfunction, which has been suggested to be associated with the disruption of PI3Kγ signaling[156]. Critically regulating the cardiac function, PI3K signaling has been heavily investigated in cardiovascular diseases, attempting to identify a promising therapeutic target in heart disease treatment.

In MI setting, the enhancement of PI3K/Akt signaling potentially offers cardioprotective effects by promoting cardiac repair. Studies on animal models carrying genetic modification on PI3K/Akt pathway and with PI3K inhibitor administration has highlighted the involvement of PI3K/Akt signaling in the regulation of post-MI cardiac function, infarct size, cardiac remodeling, and vascular repair[162-164]. Moreover, deletion of cardiac-specific GSK3β or GSK3α has protected against adverse post-MI cardiac remodeling partially by increased cardiomyocyte survival and proliferation[165,166]. However, fibroblast-specific GSK3β deletion has the opposite effects under MI, causing adverse remodeling with excess fibrosis[167]. These findings highlight the importance of understanding the cell type-specific roles of PI3K/Akt signaling as this pathway has distinct and essential functions in different cell types. In addition, PI3K/Akt signaling is of central importance mediating vascular function, orchestrating post-MI cardiac function[168]. PI3K/Akt signaling is required to support coronary angiogenesis by promoting endothelial cell survival, migration, elongation, and angiogenesis[121,169-172]. Promotion of angiogenesis via enhanced VEGF and angiopoietin-1 expression has improved cardiac functions in porcine MI hearts by increased vascular density and reduced apoptotic cells[173]. Deficiency of eNOS has rendered exacerbated post-MI cardiac dysfunction associated with decreased capillary density and cardiac hypertrophy, and endothelial-specific eNOS overexpression have attenuated cardiac dysfunction and enhanced survival after myocardial infarction in mice[174,175]. Overall, further studies should be carried out to reveal the roles of PI3K/Akt signaling in different cell types in the MI setting, which will guide PI3K/Akt-targeted pharmacological development in MI therapy.

In this dissertation, I focus on exploring the function of PI3Kα and PI3Kβ in the heart, specifically in cardiomyocytes and endothelial cells, to outline our current understanding on the roles of PI3Kα and PI3Kβ in cardiomyocytes and endothelial cells (Figure 1.3 and 1.4) and to indicate the gaps in our knowledge in this field.
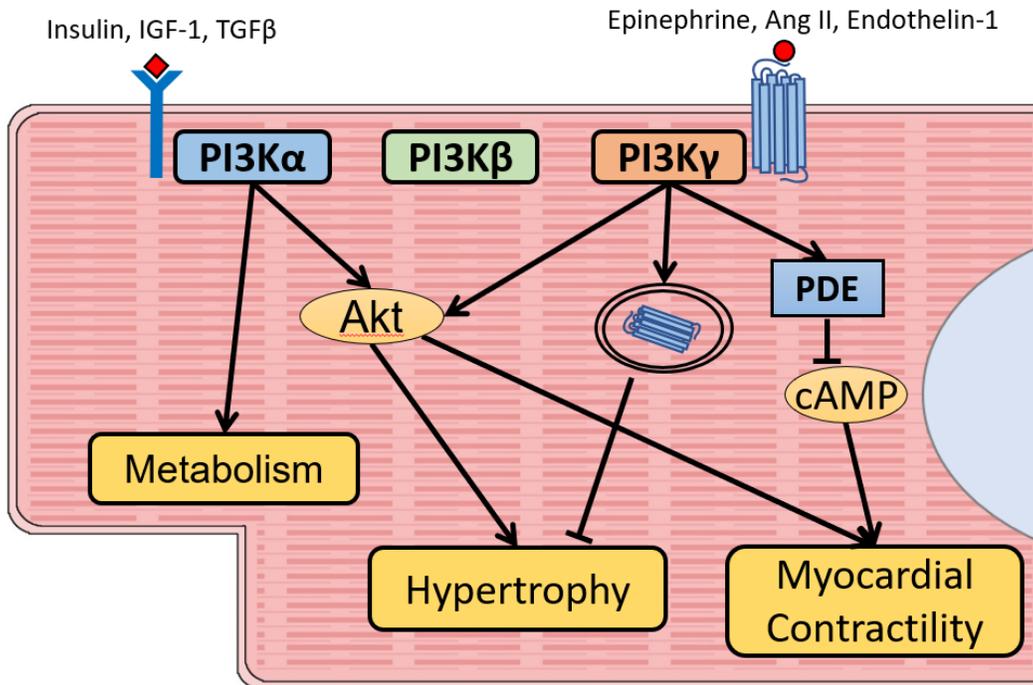
Figure 1. 3. PI3Kα, PI3Kβ, and PI3Kγ distinctly and collaboratively regulate metabolism, cardiomyocyte hypertrophy, and cardiac contractility.
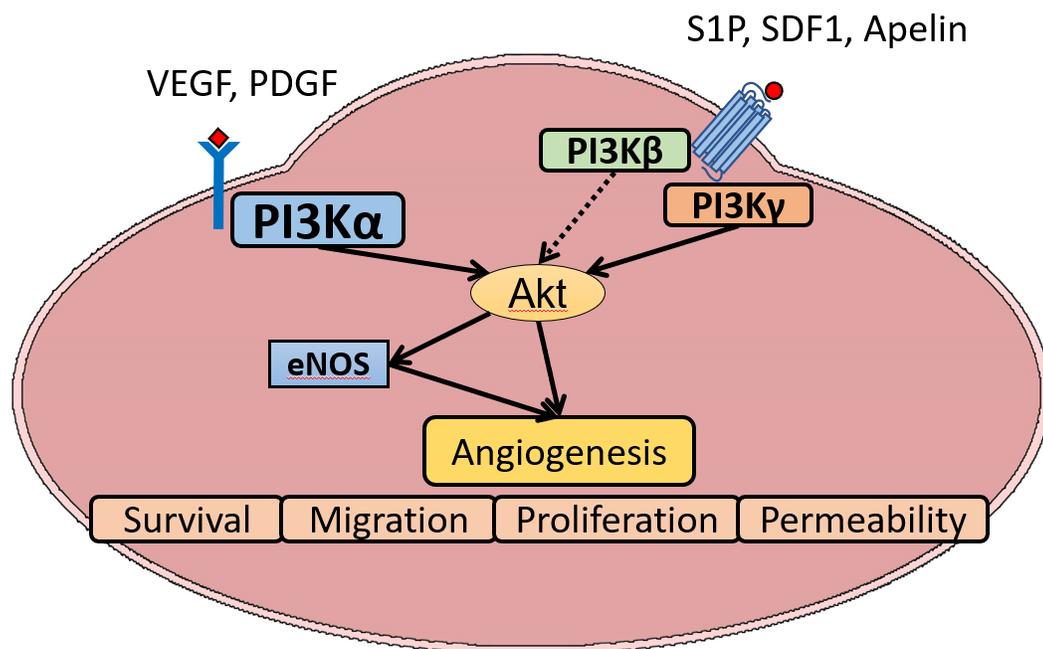
Figure 1. 4. PI3K isoforms participate in regulating angiogenic responses.

1.2.2. PI3Kα in the Heart

In cardiomyocytes, the PI3Kα/Akt signaling pathway plays a central role both in physiological and pathological conditions. Firstly, PI3Kα/Akt signaling participates in the regulation of embryonic cardiomyocyte differentiation and growth and cardiomyogenesis[176-178]. Moreover, PI3Kα/Akt pathways control postnatal cardiomyocyte size and physiological hypertrophy[121]. Transgenic mice with cardiac-specific constitutively active or dominant-negative p110α expression developed an increase or a decrease in heart size respectively as a result of different degrees in cardiomyocyte hypertrophy, highlighting the importance of PI3Kα signaling in cardiomyocyte size regulation[156,179]. Dominant-negative p110α abolishes exercise-induced cardiac hypertrophy. Beside regulating cardiomyocyte growth, PI3Kα also influences myocardial contractility, probably via maintaining the level of Ca^{2+}-handling proteins, including L-type Ca^{2+} channels, ryanodine receptors, and SERCA2a[180,181]. Furthermore, PI3Kα regulates the expression of structural components of cardiomyocytes such as dystroglycan, filamin C, and Rho-associated coiled-coil containing protein kinase 2, participating in Z-disc alignment and mechanotransduction[182]. Additionally, PI3Kα protects the heart against various cardiovascular diseases. PI3Kα/Akt/GSK3β signaling was upregulated during recovery from cardiac pacing-induced HF in a canine model[183]. PI3Kα have been demonstrated to have cardioprotective effects in dilated cardiomyopathy and pressure-overload mouse models[184,185]. And lack of PI3Kα has rendered the development of atrial fibrillation in a dilated cardiomyopathy mouse model[186]. Cardiac-targeted PI3Kα overexpression via gene therapy has mitigated diabetic cardiomyopathy by reducing oxidative stress, ER stress, and apoptosis[187]. When facing MI, studies on transgenic mice with increased or decreased PI3Kα activity have demonstrated the cardioprotective effect of PI3Kα in post-MI remodeling by regulating microRNA (miRNA) and messenger RNA (mRNA)[188].

In endothelial cells, PI3Kα is the most active PI3K isoforms followed by PI3Kβ, similar to those in many cell types[189]. It is a key player in angiogenesis, a series of fine-tuning processes involving endothelial survival, proliferation, permeability, vascular tone regulation, and vascular remodeling. Endothelial-specific inactivation of PI3Kα causes embryonic lethality due to severe vascular defects, highlighting the essentiality of PI3Kα in angiogenic sprouting and vascular remodeling[189]. It controls VEGF-dependent endothelial migration through Akt-independent RhoA and Akt-dependent eNOS activation[189,190]. Responding to PI3K/Akt activation, phosphorylation and subsequent cytosol localization of forkhead transcription factors in endothelial cells have resulted in gene expression alteration, mediating cell survival and proliferation[191]. Moreover,

PI3Kα promotes TNF-induced endothelial permeability and leukocyte transendothelial migration via the components of endothelial adherens junctions, including vascular endothelial cadherin (VE-cadherin)[192,193].

Overall, PI3Kα exerts cardioprotection against cardiovascular diseases. Thus, it is no surprise that pharmacological PI3Kα inhibition via isoform-specific inhibitor or activation by insulin has displayed opposite effects in a mouse IR model[194].

1.2.3. PI3Kβ in the Heart

The knowledge regarding the roles of PI3Kβ in the cardiomyocytes and endothelial cells are scarce. In part, this is due to the redundant function of PI3Kβ with PI3Kα and PI3Kγ. Currently, research has underestimated the participation of PI3Kβ in cardiac contractility as the lack of PI3Kβ activity in the cardiomyocytes does not affect its contractility[180]. In the endothelial cells, PI3Kβ have been demonstrated primarily responding downstream GPCR activation, triggered by SDF-1, IL-8, and sphingosine-1-phosphate (S1P) as PI3Kγ[189]. Despite that, the lack of PI3Kβ activity has been shown to decrease GPCR agonist-induced angiogenic effects, which indicates that PI3Kγ alone is not sufficient to relay GPCR-induced angiogenic signaling[189]. PI3Kβ/Akt has been shown to mediate endothelial migration by S1P which has protected the heart against IR injury in a mouse model[195,196]. Furthermore, a study using gene therapy to enhance cardiac PI3Kβ expression has highlighted that PI3Kβ has reduced cardiomyocyte apoptosis and promoted cardiomyocyte cell cycle activity after MI[197]. Thus, the currently limited understanding on PI3Kβ has suggested its involvement in cardiomyocyte survival and angiogenic signaling responding to GPCR agonists. However, further studies are required to elucidate the role of PI3Kβ in cardiomyocytes and endothelial cells.

1.2.4. PI3Kγ in the Heart

The PI3Kγ, made up of p110γ/p101 complex, exerts full functional potential following GPCR activation by direct interaction of the helical domain of p110γ and p101 with $G_{βγ}$ heterodimers[198]. Research has supported the expression of PI3Kγ in cardiomyocytes and endothelial cells, displaying divergent roles in the heart. In cardiomyocytes, PI3Kγ is required to fine-tune GPCR signaling. It acts as a negative regulator of cardiac contractility by controlling cAMP levels through kinase activity-independent scaffolding action. PI3Kγ mediates subcellular compartmentalization of cAMP through protein-protein interaction with PDE3 which degrade cAMP, resulting in the alteration of cAMP-dependent PKA activation and subsequent changes in PLB and sarcoplasmic reticulum $Ca2^+$ ATPase activity[119,156]. Thus, PI3Kγ is fine tuning the

GPCR/cAMP-induced cardiac contractility. Moreover, both PI3Kγ kinase-dependent and -independent activity are required to orchestrate GPCR signaling through regulating β-adrenergic receptor (β-AR) internalization and desensitization[199]. Activated PI3Kγ interacts with and relocates G protein-coupled receptor kinase-2 (GRK-2) which phosphorylates β-AR and eventually controls β-AR endocytosis. Both in pressure overload- and MI-induced heart failure mouse models, PI3Kγ expression was upregulated, indicating the involvement of PI3Kγ in cardiovascular disease progression[119,200]. When facing pressure overload, while the kinase activity of PI3Kγ mediates adverse remodeling via Akt and MAPK signaling, its scaffolding function are necessary to prevent adverse outcomes via reducing cAMP level[119]. However, it is a different story in the ischemic heart, which strengthens the involvement of PI3Kγ in post-MI repair via angiogenesis. Loss of PI3Kγ activity by systemic inhibitor administration or genetic modification in mice have displayed significantly deteriorated cardiac function associated with impairing reparative neovascularization and enhanced cardiomyocyte apoptosis, despite the reduced leukocyte infiltration[200]. Similarly, a study on inflammatory vascular injury has also demonstrated the importance of PI3Kγ activity in endothelial regeneration and vascular repair[201]. Hence, while the kinase-independent function of PI3Kγ is indispensable in the heart, PI3Kγ kinase activity has distinct functions in the cardiomyocytes and endothelial cells in cardiovascular diseases. Inhibition of PI3Kγ activity might exert cardioprotective effects in pressure overload-induced cardiomyopathy by suppressing PI3Kγ/Akt signaling in cardiomyocytes, while it causes devastating effects in the ischemic hearts through compromising vascular repair. Because of these, further investigations are required before targeting PI3Kγ in the clinical context, and it is likely that PI3Kγ should be targeted in a case-by-case scenario.

Besides having distinct functions in the regulation of cardiac functions, these three PI3K isoforms undeniably crosstalk with each other in cardiac health and heart diseases. First, the theories behind the coexistence of PI3Kα and PI3Kβ within the same cell type in the heart are not well demonstrated. And there is a suggestion that PI3Kα and PI3Kβ collaborate to fine-tune RTK signaling. Moreover, both being activated downstream GPCR signaling, PI3Kβ and PI3Kγ are expected to coordinate or antagonize in signaling transduction. Additionally, while the complicated crosstalk between RTK and GPCR signaling is still under investigation, there might be an interaction between PI3Kα and PI3Kγ. McMullen et al have demonstrated that PI3Kα negatively regulates pathological signaling by inhibiting pathological stimuli-triggered GPCR and PI3Kγ activation under pathological setting and subsequently provides cardioprotective effects[184]. Overall, a better understanding of the functions of these PI3K isoforms in the heart

and in cardiovascular diseases is essential to permit pursuing therapeutic strategies through manipulating PI3Ks.

1.3. Cardiac Implication of PI3K inhibitors in Anticancer Therapies

PI3K/Akt signaling amplification due to various reasons such as *Pik3ca* mutation or loss of PTEN function is the most frequent alteration in human cancers, including breast cancer, lung cancer, head and neck cancer, colorectal cancer, and others[202]. And PI3K pathway activation contributes to the acquisition of resistance to anticancer agents in various cancers. Thus, animal studies and clinical trials have been under investigation to test the therapeutic opportunity and effectiveness of PI3K signaling inhibition in cancer treatments. Pan-Class I PI3K inhibitors (e.g. Buparlisib and Pictilisib), Akt inhibitors, mTOR inhibitors, and dual-specificity PI3K/mTOR inhibitors have been developed and shown clinical activity. However, because of the diverse role of PI3K signaling in human health and the optimal therapeutic responses achieved by sufficient depth of target inhibition in tumor, treatment-related complications are commonplace in patients with systemic administration of PI3K/Akt pathway inhibitors, which includes hyperglycemia and hyperinsulinemia, hyperlipidemia, rash, and gastrointestinal discomforts[203]. Moreover, combinations of PI3K pathway inhibition and other targeted therapies such as chemotherapy are frequently required to achieve effective clinical responses, which raise the potential risks of adverse effects. Due to these reasons, the clinical efficacy of these drugs has been compromised. To maximize therapeutic efficacy and to minimize drug toxicity, PI3K isoform-selective inhibitors have been explored. Alpelisib (BYL719), a potent small-molecule PI3Kα-selective inhibitor, has demonstrated antitumor activity in mice carrying *Pik3ca*-mutated cancer graft and patients with *Pik3ca*-altered cancer[204-206]. Recently, a phase 3 trial has evidenced that the combination of PI3Kα inhibitor and an estrogen receptor antagonist has significantly prolonged progression-free survival in patients with *Pik3ca*-mutated advanced breast cancer[207]. And patients treated with PI3Kα inhibitor have manifested manageable side effects such as mild to moderate hyperglycemia and gastrointestinal reactions[205,206]. With the promising results from current clinical trials, PI3Kα inhibitors have offered exciting prospects for treating many other malignancies, especially when combined with other anticancer remedies. However, further evaluation of the potential risks and adverse effects of these agents is required.

Unlike PI3Kα, PI3Kβ has gained less attention as an anti-cancer target despite that research has indicated the potential role of PI3Kβ in PTEN loss-driven prostate cancer and human epidermal growth factor receptor 2 (HER2)-driven breast tumor development[105,113]. This

might be largely due to the limited knowledge we have obtained in the last few decades regarding PI3Kβ's function in cancer progression and in human health. Preclinical studies have demonstrated the potential of using PI3Kβ inhibitors to treat cancer with distinct genetic profiles, such as PTEN-deficient tumor and renal cell carcinoma with *Vhl* and *Setd2* mutations[208,209]. A recently published phase I clinical trial has evaluated PI3Kβ inhibitor-GSK2636771 in patients with PTEN-deficient advanced solid tumors, which suggests that it had clinical benefit in these patients with manageable side effects such as hypophosphatemia, hypocalcemia, and gastrointestinal discomforts[210]. Further clinical studies are currently underway.

However, despite the initial existing results from early clinical trials on the use of PI3K isoform-selective inhibitors, especially PI3Kα inhibitors, as cancer therapy, a concern has been raised on the likelihood of cardiotoxicity because of the critical roles of PI3Ks in the heart. For one thing, the importance of PI3Kα in cardiac health is well accepted. PI3Kα inhibition regimen contributes to cardiac implications such as Long-QT syndrome. QT interval prolongation arises from PI3K/Akt suppression associated with increased depolarizing currents and decreased repolarizing currents by regulating cardiac iron channel activity, protein expression, and/or trafficking, which is documented in diabetes mellitus and PI3K inhibitor usage[211,212]. And about 16% of patients have experienced QTc prolongation in a clinical study[213]. Generally, these effects of PI3Kα inhibitors on the heart impose a heightened risk of poor outcomes in patients with life-threatening arrhythmia. For another, my colleagues have revealed that PI3Kα is required for heart function recovery from tamoxifen-triggered cardiac dysfunction[214]. And, in conjunction with doxorubicin-a chemotherapy agent, PI3Kα inhibition has led to biventricular atrophy and severe right ventricular dysfunction[215]. These findings highlight the essential of cardiac PI3Kα when facing cardiac toxic agents, raising the awareness of potential cardiac side effects in combined cancer therapies. In addition, there are knowledge gaps in the roles of PI3Kα and P3Kβ in heart diseases development and progression, imposing the potential of PI3K isoform-specific inhibition affecting cardiac health and cardiac disease recovery.

It is no doubt that managing and minimizing potential toxicity risks on the heart are of great importance, particularly in vulnerable patients with high susceptibility to heart diseases. Effective drug combination and dosing strategies, appropriate subpopulation selection, and robust biomarker identification will help overcome toxicities and maximize therapeutic benefit. Moreover, advances in drug development, such as nanoparticle-encapsulated inhibitors, would limit adverse side effects by targeting the tumor while sparing normal organs. Besides that, a

better understanding of the roles of PI3K isoforms in the heart could improve PI3K-directed drug development and safety.

1.4. Hypotheses and Objectives

The use of transgenic mice and animal models of MI have provided great opportunities for the investigator to delineate the cellular and molecular mechanisms that contribute to post-infarct cardiac remodeling. The increasing refined insights in the functions of PI3K isoforms in cardiac health and in post-infarct cardiac remodeling will unveil new opportunities to improve the therapeutic efficacy of PI3K inhibitors in cancer and to identify therapeutic targets for treating ischemic diseases. Thus, acknowledging the gaps of PI3K isoforms in cardiac health, I am specifically investigating how PI3Kα and PI3Kβ function in endothelial cells and cardiomyocytes to modulate the cardiac function and post-infarct ventricular remodeling during my graduate study, evaluating the therapeutic possibility of targeting PI3K isoforms in ischemic treatment and potential consequences of systemic PI3Kα inhibition on cardiac health.

First, I hypothesized that PI3Kα is a critical player in the regulation of post-MI cardiac recovery both in endothelial cells and cardiomyocytes. Specifically, the following objectives have been set to test the hypothesis:

Objective 1: To characterize the effects of systemic administration of PI3Kα inhibitor-BYL719 in the heart and in post-infarct cardiac recovery in mice.

Objective 2: To evaluate the impact of conditional loss of endothelial PI3Kα in post-MI repair using endothelial PI3Kα-inactivated mice.

Objective 3: To investigate the functions of PI3Kα specifically in cardiomyocytes after MI using cardiomyocyte PI3Kα-deficient mice.

Next, I hypothesized that PI3Kβ may play a distinct role in endothelial cells and cardiomyocytes in post-MI cardiac remodeling, with the following objectives:

Objective 4: To assess the roles of endothelial PI3Kβ in cardiac health and in the ischemic heart.

Objective 5: To evaluate the influences of lacking cardiomyocyte PI3Kβ activity in a mouse MI model.

Chapter 2

Materials and Methods

2.1. Experimental Animals

Animal experiments were conducted in accordance with the Canadian Council for Animal Care guidelines, with protocols approved by the Animal Care and Use Committee at the University of Alberta.

Wildtype (C57BL/6, WT) and αMHC-Cre mice were purchased from the Jackson Laboratory. BYL719 (Chemietek) or vehicle was given to 11- to 12-week-old WT mice in the morning for 10 days (50 mg/Kg/day, *p.o.*). Mice with cardiomyocyte-specific PI3Kα inactivation (p110α-αMHC) controlled by α-MHC promoter were generated by crossbreeding p110α$^{flx/flx}$ (p110αFlx) mice with αMHC-Cre mice, while mice without endothelial cell PI3Kα activity (p110αTie2) were generated by crossbreeding p110αFlx with Tie2-MerCreMer mice under the control of Tie2 promoter-driven conditionally-active Cre-recombinase[189]. Littermates from each genotype, p110αFlx, are used as controls. Tamoxifen (Sigma-Aldrich) was given to p110αTie2 and its littermates at 80 mg/kg/day for 5 days via intraperitoneal (IP) injection when they were about 10 weeks old. p110αFlx are mice with floxed exons 18 and 19 in *Pik3ca* which are critical in the regulation of kinase activity[189]. Cre-recombinase activation deletes exons 18 and 19, producing a truncated p110α protein without kinase activity. The generation of cardiomyocyte (p110β-αMHC) or endothelial (p110βTie2) specific PI3Kβ inactivated mice was by crossbreeding p110β$^{flx/flx}$ (p110βFlx) with αMHC-Cre or Tie2-MerCreMer mice[94,214,216]. Homozygous littermates p110βFlx mice were used as control. Tamoxifen was given to 10-week-old p110βTie2 and littermate controls at 80 mg/kg/day for 5 days via IP injection to activate Cre in endothelial cells in p110βTie2. Cre-recombinase deletes exons 21 and 22 from *Pik3cb*, producing a truncated p110β which lacks catalytic activity[94,217]. All tamoxifen-treated mice were given a 2-week washout period before experiments. All mice used in the experiments were on C57BL/6 background, and they were viable with no apparent phenotypes under our husbandry conditions.

Polymerase chain reaction (PCR) analysis of the genomic DNA from the aforementioned mice was carried out to detect floxed *Pik3ca* or *Pik3cb*, Tie2 transgene, Cre transgene, and partial-deleted *Pik3ca* or *Pik3cb* as previously described[94,189]. Specifically, WT and floxed *Pik3ca* allele were amplified with primers 5'-GGATGCGGTCTTTATTGTC-3' and 5'-TGGCATGCTGCCGAATTG-3'. Primers 5'-AGTGAACGCTATGCATCACACCAGC-3' and 5'-AAGTACAAACATCCAAGCAA-3' were used to detect WT and floxed *Pik3cb* allele. PCR for the Cre transgene was performed using primers 5'-GCGGTCTGGCAGTAAAAACTATC-3' and 5'-GTGAAACAGCATTGCTGTCACTT-3'. The Tie2 transgene was detected with primers 5'-

CGAGTGATGAGGTTCGCAAG-3' and 5'-TGAGTGAACGAACCTGGTCG-3'. The floxed *Pik3ca* allele following Cre-mediated deletion was detected in genomic DNA prepared from the hearts (p110α-αMHC and controls) or the lungs (p110αTie2 and controls) using primers 5'-ACACACTGCATCAATGGC-3' and 5'-GCTGCCGAATTGCTAGGTAAGC-3'. The sequences of the primers detecting the partially deleted Pik3cb allele in the hearts from p110β-αMHC and controls and in the lungs from p110βTie2 and controls are 5'-ACAGCCATGAGACCCCACTT-3' and 5'-AAGTACAAACATCCAAGCAA-3'.

2.2. Human Explanted Hearts

Non-failing control human heart and failing post-MI adult human heart tissue were collected as part of the Human Organ Procurement and Exchange program (HOPE) and Human Explanted Heart Program (HELP) respectively. Informed and signed consents were obtained from all participants. The collection of human cardiac tissues and ethics were approved by the Mazankowski Alberta Heart Institute and the Institutional Ethics Committee.

Once the hearts were arrived, they were quickly dissected into infarct, peri-infarct, and non-infarct areas based on the structural differences between tissues, which were flash-frozen immediately in liquid nitrogen for further molecular analyses or embedded in optimal cutting temperature compound (OCT) for histological analysis. All tissue was preserved in the -80°C freezer.

2.3. *In Vivo* myocardial infarction and ischemia reperfusion Models

MI achieved by permanent ligation of the proximal left anterior descending artery (LAD) or sham surgery was performed on 12-week-old male mice by a technician who was blinded to the genotype as described[218]. Briefly, a left thoracotomy was performed on anesthetized and intubated mice in the fourth intercostal space. After opening the pericardium and exposing the left ventricle, LAD was identified and encircled with a 7-0 silk. LAD was ligated in MI mice, while it was encircled only in sham-operated mice. Afterwards, the muscle and skin were closed in layers with a 6-0 silk suture. Animals were inspected at least twice daily before sacrificed, and survival data were recorded. We performed autopsy on each mouse found dead during the study. At indicated time points after surgery, mice were anesthetized and hearts were quickly excised and dissected into infarct, peri-infarct, and non-infarct areas, which were flash-frozen immediately in liquid nitrogen for further molecular analyses. For histological analysis, hearts were fixed in 10% formalin or embedded in optimal cutting temperature compound (OCT).

Infarct size was visualized using Triphenyl Tetrazolium Chloride (TTC) (Sigma) staining and Masson's trichrome staining.

The procedures for IR surgery were similar to the MI surgery except that a piece of polyethylene tubing was placed and the suture was tightened around the LAD and tubing to minimize vessel damage[219]. The LAD artery was occluded for 30 minutes before the removal of the tubing and the suture to achieve reperfusion. The IR surgery protocol was validated using Evans blue perfusion and non-invasive electrocardiogram (ECG).

2.4. Echocardiographic Measurements

At day 7 or week 4 after the surgery, mice were anesthetized to undergo noninvasive transthoracic echocardiography using a Vevo 3100 high-resolution imaging system (Visualsonics, Toronto, Canada). Standard parasternal longitudinal and short-axis views were obtained. All echocardiographic images were digitally stored, and offline analysis was performed on a workstation installed with VevoLab software. Conventional measurements were carried out to evaluate cardiac systolic and diastolic function. Left ventricular systolic function parameters, including left ventricular end-systolic volume (LVESV), left ventricular end-diastolic volume (LVEDV), ejection fraction (EF), stroke volume (SV), and fractional area change (FAC), were obtained according to the manufacturer's manual. The wall motion score index (WMSI) was assessed based on 17-segment LV model, and wall motion was scored as described[218,220].

Speckle-tracking based strain analysis on 2-dimensional echocardiographic images acquired from the longitudinal views was carried out[221]. In brief, semi-automated tracing of the endocardial and epicardial borders was achieved over 3 cardiac cycles, and then tracked images were further processed for strain measurements. Each long-axis view of the LV was divided into 6 standard anatomic segments (anterior-base, anterior-mid, anterior-apex, inferior-base, inferior-mid, and inferior-apex). Peak systolic strain or longitudinal strain was obtained from all 6 standard segments, and global peak systolic strain or global longitudinal strain was calculated as the average of 6 segments.

2.5. Blood Glucose and Body Composition Measurements

Blood glucose was measured in overnight-fasted mice from a tail vein prick using the Ascensia Contour Blood Glucose Monitoring System (Bayer). Body composition, including fat mass and lean mass, was detected in live and conscious mice using an NMR-MRI scanner.

2.6. Immunofluorescence

Immunofluorescence staining was performed on formalin-fixed paraffin- or OCT-embedded tissue using established protocols[218]. Briefly, paraffin slides were deparaffinized and redehydrated with alcohol grades before antigen retrieval process, while the frozen sections were fixed with 4% paraformaldehyde and rehydrated in wash buffer. Then, permeabilization with 0.1% triton X-100 and blockage with 4% Bovine Serum Albumin were carried out in sequence. The sections were incubated overnight at 4°C with a primary antibody, including anti-mouse Ly-6B.2 (AbD Serotec), anti-mouse CD68 (AbD Serotec), anti-p110β (Santa Cruz), anti-CD31 (BD Pharmingen), and anti-Ki67 (AbD Serotec), followed by incubation with secondary antibody at 37°C for 1 hours. After mounting antifade mounting mediate with DAPI (Life Technologies), the slides were used for visualization and imaging through fluorescence microscopy (Olympus IX81). Wheat Germ Agglutinin (WGA, Alexa Fluor 488 conjugate, Invitrogen) or fluorescent conjugated phalloidin staining for F-actin (Invitrogen) was performed to identify the cardiomyocytes through incubation on slides for 30 minutes at room temperature followed by mounting with DAPI media.

Fluorescein-conjugated Ricinus Communis Agglutinin I (RCA 1; Lectin; Vectorlabs) intravital perfusion method was used to identify the functional vasculature as described[218]. Lectin (0.2 mg/100 µl Saline) was injected into the anesthetized mice's circulation via the right jugular vein and circulated vascular system for 18 minutes, followed by papaverine HCL (0.2 mg/50 µL Saline; Sigma Aldrich) intravenous injection for two minutes. Then, the hearts were quickly excised and fast-frozen in OCT. Tissue sections were fixed with 4% paraformaldehyde and rehydrated using PBS buffer. Mounted with DAPI media, the sections were visualized and imaged.

Fragmented DNA of apoptotic cells was detected using the DeadEnd Fluorometric Terminal Deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick-End Labeling (TUNEL) System (Promega) according to the manufacturer's instructions. The heart sections were incubated with buffer containing equilibration buffer, nucleotide mix, and recombinant TdT at 37 °C for 1 hour, followed by mounting the sections with DAPI media and visualization by fluorescence microscopy. All the fluorescent images were analyzed using Metamorph software (Version 7.7.0.0).

2.7. Endothelial Cell Culture and Reagents

Human umbilical vein endothelial cells (HUVECs) and human coronary artery endothelial cells (HCAECs, ATCC) were used in this study. HUVECs were cultured in M199 (Life Technologies) containing 20% fetal bovine serum (FBS) and 1% endothelial cell growth supplement (VWR) using established protocols[222], while HCAECs were cultured in vascular cell basal medium (ATCC) with endothelial growth kit-VEGF (ATCC), supplemented with VEGF, fibroblast growth factor (FGF), epidermal growth factor (EGF), IGF, ascorbic acid, and 5% FBS (ATCC) at 37°C in a humidified atmosphere with 5% CO_2. When indicated, endothelial cells were cultured in serum- and growth supplement-free medium in a hypoxic chamber with 1% O_2 and 5% CO_2. Endothelial cells were used between passage 3 to 7. Cells cultured to 70-80% confluence in 6-well plates were transfected with small interfering RNA against human p110β (sip110β) or scrambled small interfering RNA (s-siRNA) in Opti-MEM (Gibco) containing Lipofectamine (Invitrogen) for 48 hours to test the effects of genetic ablation of p110β on endothelial cells. Pharmacological inhibition of PI3Kα or PI3Kβ using the PI3Kα-specific inhibitor BYL719 (Cayman Chemical) at the indicated concentration or PI3Kβ-specific inhibitor TGX-221 (500 nmol/L, Cayman Chemical) was used on 70-80% confluent endothelial cells. Cells were serum-starved in basal medium without growth factors and FBS for 5 hours prior to stimulation with 50 ng/ml or 100 ng/ml recombinant human $VEGF_{165}$ (VEGF, PeproTech Inc) for 10 minutes. In addition, the Akt inhibitor MK-2206 (1 µmol/L, APExBIO) was used to inhibit Akt activity for 1 hour before VEGF stimulation.

2.8. Murine Bone Marrow Cell Isolation

Bone marrow cells of p110β-Tie2 and littermate control mice were isolated from the femur bones as previously described[223]. Briefly, after anesthetized (2% isoflurane with O_2) a mouse, the femur was dissected from surrounding tissue. Then, the proximal and distal end of the bone was trimmed to expose the interior marrow shaft. Using a 28G needle and a 1-ml syringe, the bone was flushed with HBSS solution. The collected cell suspension was centrifuged at 250xg for 10 min at room temperature. The pelleted bone marrow cells were flash-frozen immediately in liquid nitrogen for further analyses.

2.9. Bead Angiogenesis Assay

In vitro angiogenesis bead assay on HUVEC or HCAECs was performed as described[218]. Briefly, HUVECs or HCAECs were treated with TGX-221, BYL719, or MK-2206 prior to being mixed with beads (Invitrogen) and incubated for 2 hours. Then the beads were

resuspended in fibrinogen solution mixed with aprotinin (0.15 Units/ml) and thrombin (0.625 Units/ml). After 18-hour incubation, Calcein AM was added to the wells for 15 minutes to label viable cells. Images were captured using a fluorescent inverted microscope (Leica). The number of sprouts and the length of sprouts per bead were analyzed using ImageJ software, and at least 20 beads per group were analyzed.

2.10. BrdU incorporation assay

Endothelial cell proliferation was evaluated using BrdU (5-bromo-2'-deoxyuridine, Abcam) incorporation assay. After being incubated with 10 µmol/L for 5 hours, cells were washed with ice-cold PBS followed by fixation in 4% paraformaldehyde. Then cell DNA was denatured by 2 mol/L hydrochloric acid at 37°C for 10 minutes with subsequent neutralization with Borate buffer (0.1 mol/L). After that, immunofluorescence staining for BrdU and DAPI was carried out.

2.11. Flow Cytometric Analysis of Endothelial Cell Death

Endothelial cell survival and death were assessed using Annexin V and propidium iodide (PI) Kit (Invitrogen) detected by flow cytometry. Cells were cultured in 6-well plates for 2 days before experiments. After being cultured with indicated treatment for overnight, endothelial cells were rinsed with ice-cold PBS and collected using Accutase Cell Detachment Solution (EMB Millipore). After washed, cells were resuspended in 100 µl binding buffer. Then, Alex Fluor 488 annexin V and red-fluorescent PI nucleic acid binding dye were added and incubated in the dark at room temperature for 15 minutes. After staining, cells were analyzed by flow cytometry using the Attune NxT Acoustic Cytometer and the Attune NxT Software (Invitrogen). Apoptotic cells show green fluorescence, necrotic cells display red fluorescence, and viable cells have little or no fluorescence.

2.12. Adult Mouse Cardiomyocyte Isolation, Culture, and Stretching

Adult murine left ventricular cardiomyocytes were isolated, cultured, and stretched as described[224,225]. Briefly, after anesthetized and heparinized a mouse, the heart was quickly excised and cannulated on a Langendorff perfusion apparatus. Then, a calcium-free buffer was perfused in the heart, followed by collagenase digestion. The digested ventricles were teased into small pieces and gently pipetted with an increasing concentration of $CaCl_2$. Then, the cells were plated in minimum essential media (MEM, Gibco) with 5% FBS for 2 hours, followed by cultured in MEM with 1% bovine serum albumin (BSA) for 1 hour. In normoxic groups, cardiomyocytes were cultured with full medium in a humidified chamber with 2% CO_2, while

hypoxic treatment was achieved by culturing the cells in serum-free medium in a chamber with low O_2 levels and 2% CO_2. When indicated, cultured cardiomyocytes were cyclically stretched at 1Hz and an elongation of 5% for 3 hours with Flexcell FX-5000 Tension System (Flexcell International Corp). Plated cardiomyocytes were cultured with vehicle, BYL719 at indicated dose, or PI3Kγ inhibitor-AS252424 at 400 nmol/L for one hour before protein collection. After completion of the experiment, cardiomyocytes were imaged under a light microscope to assess the cell viability. Culture media were collected to evaluate cell death by examining creatine kinase activity (BioAssay Systems), and cardiomyocytes were fixed for TUNEL staining.

2.13. Immunoblotting and Nuclear Fractionation

Extracted proteins from left ventricles, endothelial cells, and isolated cardiomyocytes were separated by 8% to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by electrotransfer to polyvinylidene difluoride membranes. Primary antibodies, including phospho-(Thr308)/phospho-(Ser473)/total Akt, phospho-(Thr202/Tyr204)/total p44/42 MAPK (Erk1/2), phospho-eNOS (Ser1177)/eNOS, phospho-(Ser9)/total GSK-3β, PI3K p110β, PI3K p110α, FoxO1, FoxO3a, Caspase 3, Caspase 8, Bak, Bax, RIP1, and RIP3 (Cell Signaling Technology), were examined. Blots were visualized and analyzed using ImageQuant LAS 4000 (GE Healthcare).

Nuclear fractionation was performed as described, with slight modifications[226]. Left ventricular tissues were homogenized in STM buffer (250 mmol/L sucrose, 50 mmol/L Tris-HCl pH 7.4, 5 mmol/L $MgCl_2$, and protease and phosphatase inhibitor cocktails). The homogenate was maintained on ice for 30 minutes and then centrifuged at 800g for 15 minutes. The supernatant was centrifuged at 800g for 10 minutes and the final supernatant was saved as the cytosol fraction. The first pellet from centrifugation was washed using STM buffer to remove excess cell debris. Then, the washed pellet was resuspended in NET buffer (20 mmol/L HEPES pH 7.9, 1.5 mmol/L $MgCl_2$, 0.5 mol/L NaCl, 0.2 mmol/L EDTA, 20% glycerol, 1% Triton-X-100, protease and phosphatase inhibitors). Centrifuged at 9,000 g for 30 minutes, the resultant supernatant was kept as the nuclear fraction. Immunoblotting was performed, and antibodies for the nuclear protein marker-Histone H3 (Cell Signaling Technology) and the cytosol protein marker-GAPDH (Santa Cruz) were used to assess the purity of the fractions.

2.14. RNA Sequencing

RNA isolation and RNA-seq were performed as described[227]. Total RNAs from left ventricles (3 left ventricles/group) were extracted using TriZol (Invitrogen) according to the

manufacturer's instructions. RNA sequencing was carried out using the Illumina HiSeq platform, and raw RNA sequencing data were analyzed with BaseSpace using the TopHat Alignment application. Significantly differentially expressed genes were determined by a false discovery rate >0.05 using the Cufflinks Assembly and DE application. Pathway enrichment analysis on significantly altered genes was performed using WebGestalt (WEB-based Gene SeT Analysis Toolkit), including KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway maps. RNA-seq data were also entered into Protein ANalysis THrough Evolutionary Relationships (PANTHER) classification system (Pantherdb.org) to categorize altered genes and their proteins. Upregulated genes involved in biological processes were subcategorized to generate a pie chart and pathway alteration was analyzed according to the PANTHER system. RNA-seq data Ingenuity Pathway Analysis was used to predict the activation and inhibition of upstream transcription factors.

2.15. TaqMan Real-time PCR

RNA expression levels were examined by TaqMan real-time polymerase chain reaction (RT-PCR) as described[218]. Total RNA was extracted from left ventricular tissue using TRIxol reagent (Invitrogen) according to the manufacturer's protocol. Complementary DNA (cDNA) from RNA was used to be amplified through PCR on a LightCycler 480 II system (Roche). The expression levels of atrial natriuretic peptide (*Anp*), brain natriuretic peptide (*Bnp*), and β-myosin heavy chain (*β-Mhc*) were determined using specific TaqMan primers and probes. The *18s* expression level was used as a control.

2.16. Statistical Analysis

Statistical analyses were carried out using SPSS Statistics 24 software, and statistical significance was defined as $p<0.05$ (two-sided). Continuous data were presented in scatter plots with mean±SEM. The differences between two independent groups were evaluated using independent t-test, or Mann-Whitney U test after normality examination. Paired t-test was carried out for two paired groups. One-way ANOVA or Kruskal-Wallis test with pairwise comparisons were used in studies with more than two groups based on the normality of the data. Two-way ANOVA was used to compare the differences between groups with two independent variables. Repeated measures ANOVA was carried out in data with multiple measures. Categorical data were compared using Fisher exact test. Survival data were presented as the Kaplan-Meier plots, and the log-rank test was used to evaluate the statistical significance.

Chapter 3

PI3Kα Inhibition Leads to Impaired Cardiac Healing and Adverse Cardiac Remodeling After Myocardial Infarction

PI3Kα Inhibition Leads to Impaired Cardiac Healing and Adverse Cardiac Remodeling After Myocardial Infarction

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3.1. Introduction

The phosphoinositide 3-kinases (PI3Ks) are conserved lipid kinases that regulate critical intracellular signaling pathways including cell growth, survival, proliferation, and metabolism. Containing one of the four 110-kD catalytic subunits (p110α, p110β, p110δ, and p110γ), the members of Class I PI3K function non-redundantly and coordinately in the regulation of cellular functions. Ubiquitously expressed and being the dominant PI3K isoform in many cell types, PI3Kα is a critical regulator in a wide range of cellular processes upon receptor tyrosine kinases (RTKs) activation. It converts phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P_2 or PIP_2) to phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3 or PIP_3) which leads to the phosphorylation and activation of Akt at Thr308. Consequently, Akt phosphorylates downstream effectors and regulates various pathways, including mammalian target of rapamycin complex 1, glycogen synthase kinase 3β (GSK3β), and endothelial nitric oxide synthase (eNOS). Indisputably, PI3Kα exerts critical roles in different cell types mediating cell survival and cell cycle, insulin signaling and metabolism, and endothelial function[106,189,214]. Because of the diverse roles of PI3Kα, the aberrations of PI3Kα are associated with a broad spectrum of human diseases. The most noticeable one is cancer. *Pik3ca*, the gene that encodes p110α, is frequently mutated or amplifying in common human tumors such as lung, head and neck, breast, endometrial, and cervical cancer[228]. In addition, disruptions of PI3Kα signaling contribute to obesity, diabetes, overgrowth disorders, and other diseases[229,230].

Currently, several PI3Kα inhibitors are under development and have been explored in human diseases. The most popular one-BYL719 (Alpelisib) has been demonstrated to have a dose- and time-dependent PI3Kα inhibition with a tolerably and safe profile in both animal and human studies[204,206,231,232]. Clinical trials have explored and supported the use of BYL719 in *Pik3ca*-related solid tumors and overgrowth syndrome[205,206,233]. Moreover, a lower dose of BYL719 has shown potential in weight control in obese mice; besides, inhibition of PI3Kα activity via butein has also demonstrated beneficial effects in weight gains and metabolic profiles in diet-induced obese mice[234,235]. Another PI3Kα inhibitor-PIK75 can reduce injury-induced arterial thrombus formation in mice by inhibiting vascular smooth muscle cell proliferation while sparing endothelial cells, suggesting the potential of developing PI3Kα inhibitor-eluting stents to tackle restenosis[236].

However, with growing evidence supporting BYL719 remedy in several diseases, little is known about the effects of PI3Kα inhibition in ischemic heart disease (IHD) which is the leading cause of deaths and years of life lost in the globe, followed by neoplasms[2]. Following myocardial infarction (MI), cardiac repair and remodeling are the results of orchestration of

various cell types such as cardiomyocytes, inflammatory cells, fibroblasts, and endothelial cells, defining the cardiac function and patient outcomes[237]. Previously, we and others have identified PI3Kα as a regulator in physiological hypertrophy, insulin signaling, and contractility in cardiomyocytes[180,214,238]. Transgenic mice with constitutively active or dominant-negative PI3Kα in cardiomyocytes have shown the cardioprotective effect of PI3Kα against MI through microRNA and mRNA regulation[188]. Moreover, studies on endothelial PI3Kα have demonstrated the dispensable role of PI3Kα in endothelial cell migration, vascular tone regulation, and angiogenesis[189,222]. Despite the accumulating knowledge of PI3Kα, the impact of PI3Kα inhibition on cardiac remodeling after MI requires thorough investigation as which will provide guidance on the use of PI3Kα inhibitors in human diseases and the therapeutic potential of PI3Kα modulation in IHD.

In the present study, we showed that BYL719 administration in mice is associated with common effects such as weight loss, hyperglycemia, and prolonged QT interval. For the first time, we reveal that BYL719 intake compromises systolic function at baseline depicted by lower left ventricular longitudinal strain and deteriorates cardiac remodeling after MI characterized by inhibited ischemia-related vascular repair and increased cardiomyocyte apoptosis. In addition to the pharmacological approach, we also used tamoxifen-inducible endothelial-PI3Kα inactivated (p110αTie2) and cardiomyocyte-specific PI3Kα inactivated (p110α-αMHC) mice to confirm the detrimental effect of loss of PI3Kα activity in endothelial cells and cardiomyocytes in post-MI cardiac remodeling. Furthermore, endothelial cell culture supports that PI3Kα is the key factor driving endothelial survival, proliferation, and angiogenesis, whilst adult murine cardiomyocyte isolation highlights the impact of PI3Kα inhibition in cell survival. These studies raise the awareness of the potential harm of PI3Kα inhibitor usage under MI condition and the therapeutic potential of PI3Kα amplification in MI treatment.

3.2. Methods

Detailed methods used in the present study are available in **Chapter 2 Materials and Methods**.

3.2.1 Animal Models and Human Explanted Hearts

All animal studies were conducted according to the Canadian Council for Animal Care guidelines and approved by Animal Care and Use Committee at the University of Alberta. Wild type (WT) C57BL/6 mice were purchased from Jackson Laboratory. BYL719 (Chemietek) or

vehicle was given to 11- to 12-week-old WT mice in the morning for 10 days (50 mg/Kg/day, *p.o.*). Inducible endothelial cell-specific PI3Kα-inactivated mice (p110αTie2) were generated by crossbreeding p110αFlx mice, displaying *Pik3ca* gene (encoding p110α) with floxed 18 and 19 exons[189], with tamoxifen-induced and Tie2 promoter-controlled Cre expression mice. Tamoxifen (80 mg/kg/day, Sigma-Aldrich) was given to 10-week-old mice by intraperitoneal injection for 5 days to induce endothelial-specific, floxed-exon deletion in p110αTie2, and this method has previously shown sparing hematopoietic cells from targeted gene deletion[239]. Cardiomyocyte-specific PI3Kα-inactivated mice (p110α-αMHC) were generated by crossbreeding p110αFlx with αMHC-driven Cre mice[214]. Homozygous male littermates p110αFlx were used as control.

Myocardial infarction was achieved by permanently ligating the proximal left anterior descending artery (LAD) on WT mice after receiving 3 doses of BYL719 or vehicle and on about 12-week-old p110αTie2, p110α-αMHC, and control mice[218]. Surgery was performed by a technician who was blinded to the mouse strains. Cardiac tissue collection was performed on ketamine (100 mg/kg) and xylazine (10 mg/kg) cocktail euthanized mice.

Human cardiac specimens from non-failing control (NFC) and failing post-MI hearts were obtained as part of the Human Organ Procurement and Exchange program (HOPE) and Human Explanted Heart Program (HELP) respectively, approved by the Mazankowski Alberta Heart Institute and the Institutional Ethics Committee at the University of Alberta.

3.2.2. Echocardiography and Electrocardiogram

Noninvasive transthoracic echocardiography was performed on mice anesthetized with 1.5% isoflurane using Vevo 3100 (Visualsonics). Conventional measurements and speckle-tracking strain analysis was carried out as previously described[239]. Non-invasive electrocardiogram (ECG) was carried out to evaluate the electrical activity of the heart as described[239].

3.2.3. Endothelial Cell Culture, Flow Cytometry, and Bead Angiogenesis Assay

Human umbilical vein endothelial cells (HUVECs) and human coronary artery endothelial cells (HCAECs, ATCC) were used between passage 3 to 7. BYL719 was used at indicated doses; and PI3Kγ inhibitor AS252424 (AS) was used at 400 nmol/L. Recombinant human vascular endothelial growth factor (VEGF$_{165}$, PeproTech) was added to stimulate endothelial responses at 50 ng/ml. Cell survival was examined on overnight vehicle- or BYL719-treated cells using flow cytometry (Attune NxT, Invitrogen) with annexin V and propidium iodide staining

(Invitrogen). Endothelial cell proliferation was evaluated using BrdU incorporation assay (Abcam). *In vitro* angiogenesis bead assay was performed on HUVECs and HCAECs as previously described[239].

3.2.4. Adult Murine Cardiomyocyte Isolation, Culture, and Stretching

Adult murine ventricular cardiomyocytes were isolated from 2% isoflurane-anesthetized mice. Plated cardiomyocytes were culture with vehicle or BYL719 for 1 hour under normoxia (2% CO_2) or hypoxia (1% O_2, 2% CO_2) before protein collection. Cyclical mechanical stretch of cardiomyocytes was achieved using Flexcell FX-5000 Tension System (Flexcell International Corp) at 1Hz and 5% elongation for 3 hours in serum-free culture medium under hypoxic atmosphere[239]. Then, cells were collected for the morphologic study.

3.2.5. Immunofluorescence and Immunoblotting

Immunofluorescence staining was performed using established protocols[239]. Antibodies against CD31, Ly6B, CD68, Ki67, and BrdU were used. Fluorescein-conjugated Lectin intravital perfusion was performed to identify the functional vasculature. Wheat Germ Agglutinin (WGA) or phalloidin were performed to identify cardiomyocytes. Fragmented DNA of apoptotic cells was detected using the DeadEnd Fluorometric Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-End Labeling (TUNEL) System.

Immunoblotting for various proteins was performed on left ventricular homogenates or cultured cell lysates as before[239].

3.2.6. Statistical Analysis

Statistical analyses were carried out using SPSS Statistics 24 software, and statistical significance was defined as $p < 0.05$ (two-sided). Continuous data were presented in scatter plots with mean±SEM. The differences between two independent groups were evaluated using independent t-test or Mann-Whitney U test after normality examination. Paired t-test was carried out for two paired groups. One-way ANOVA or Kruskal-Wallis test with pairwise comparisons was used in studies with more than two groups based on the normality of the data. Two-way ANOVA was used to compare the differences between groups with two independent variables. Repeated measures ANOVA was carried out in data with multiple measures. Categorical data

was compared using Fisher exact test. Survival data were presented as the Kaplan-Meier plots, and the log-rank test was used to evaluate the statistical significance.

3.3. Results

3.3.1. Inhibition of PI3Kα with BYL719 Results in Subtle Cardiac Dysfunction and Systemic Disturbances in Mice

We first examined the influence of pharmacological PI3Kα inhibition on WT mice by orally and daily giving mice 50 mg/kg BYL719 for 10 days (Figure 3.1A). This treatment was chosen because previous studies have confirmed the therapeutic effects of this strategy on mice bearing PI3Kα-driven tumors, osteosarcoma, lung cancer, and squamous cell carcinomas[97,204,240,241]. We demonstrated that BYL719 suppressed daily weight gain, leading to a decrease in body weight at day 10 (Figure 3.1B). Examining the effect of BYL719 on blood glucose, we demonstrated that BYL719 administration transiently increased blood glucose without affecting fasting glucose over 10 days (Figure 3.1C). Moreover, BYL719-treated mice displayed an increase in fat mass and a decrease in lean mass over 10 days (Figure 3.1D). These findings indicate the influence of PI3Kα inhibition in glucose metabolism and body composition.
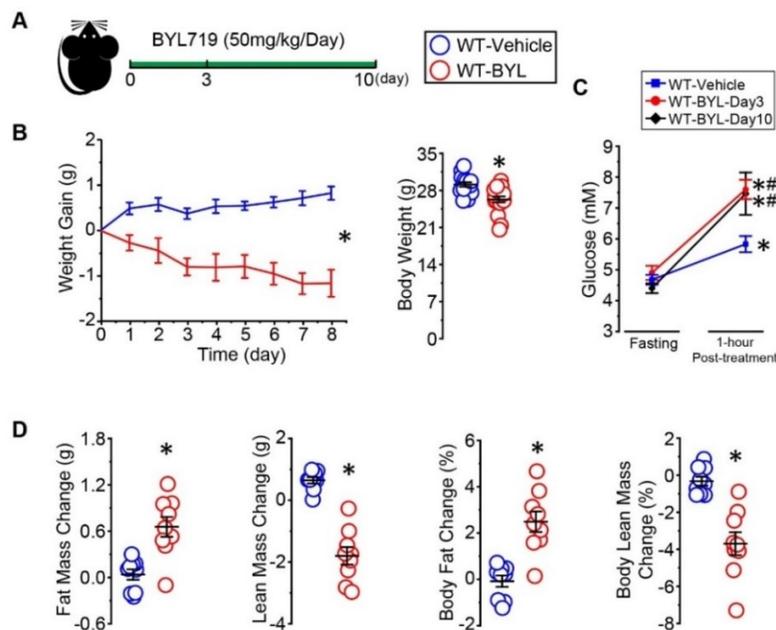
Figure 3. 1. Administration of PI3Kα inhibition BYL719 leads to a decrease in body weight, transient hyperglycemia, increased fact mass, and reduced lean mass in mice. (A) Schematic diagram for the experiment. **(B)** Daily body weight changes and body weight at day 10 in mice. **(C)** Blood glucose levels measured in mice. **(D)** Body composition alteration in control and BYL719-treated mice. *$P<0.05$ vs indicated group in B and D and vs fasting in C; #$P<0.05$ vs WT-Vehicle in C.

Next, we looked at how BYL719 affects the heart. BYL719 treatment was associated with reduced heart and left ventricle weight (Figure 3.2A). ECG examination showed prolonged QT interval in BYL719-treated mice, without affecting heart rate, PR interval, and QRS duration (Figure 3.2B). Protein level examination on left ventricular lysates surprisingly showed a significant decrease in eNOS phosphorylation with BYL719 treatment, despite that the levels of p110α, phosphorylated Akt, and phosphorylated GSK3β were comparable (Figure 3.2C). Furthermore, despite the normal ejection fraction present on BYL719-treated mice, strain analysis on echocardiography indicated significantly lower global longitudinal strain in BYL719-treated mice, suggesting the existence of subtle systolic dysfunction (Figure 3.2D).

Taken together, these results establish that PI3Kα inhibition with BYL719 has systemic effects in mice. Besides affecting the body composition and glucose metabolism, PI3Kα inhibition results in decreased heart weight, prolonged QT interval, and subtle cardiac dysfunction.
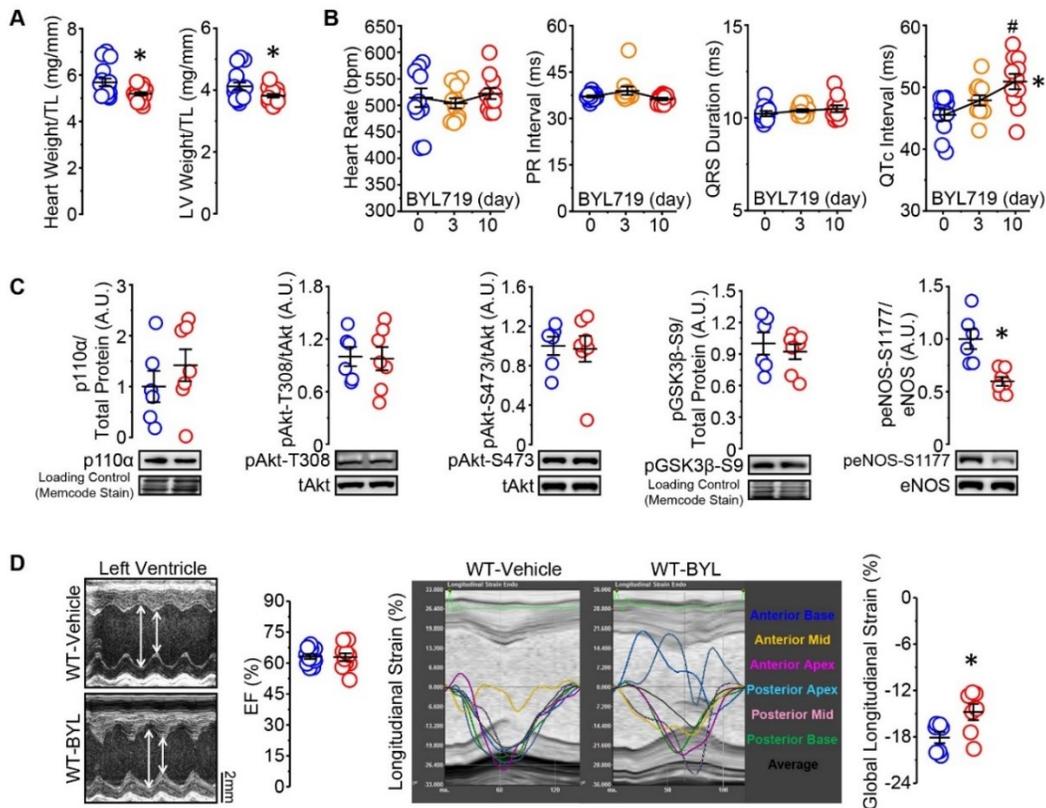
Figure 3. 2. PI3Kα inhibition results in reduced heart weight, prolonged QTc interval, and subtle systolic dysfunction. (A) Heart weight and left ventricular (LV) weight changes in mice. **(B)** ECG characteristics with BYL719 administration. **(C)** Protein levels of p110α, Akt, GSK3β, and eNOS in LV lysates. **(D)** Ejection fraction and global longitudinal strain in control and BYL719-treated mice. *P<0.05 vs indicated group in A, C, and D and vs BYL effect in B; #P<0.05 vs day 0 in B.

3.3.2. BYL719 Administration Is Detrimental to Post-MI Cardiac Repair

To discern the function of PI3Kα under MI condition, we first examined the protein levels of p110α and Akt phosphorylation in post-MI human and mouse hearts. Compared with non-failing human hearts, left ventricular specimens from MI patients showed a markedly increase in p110α level both in the infarct and peri-infarct regions, accompanied by different degrees of enhanced Akt phosphorylation among regions (Figure 3.3A). Moreover, a similar tendency of p110α/Akt signaling alterations was documented in post-MI mouse hearts (Figure 3.3A).

We next investigated whether pharmacological PI3Kα inhibition would impact MI outcomes (Figure 3.3B). Post-MI survival rate was slightly decreased in BYL719-treated mice compared with controls, whilst the causes of death due to cardiac rupture or not were comparable between groups (Figure 3.3C). These findings suggest that arrhythmia-related sudden cardiac death might not be a major player in the death of post-MI BYL719-treated mice. Echocardiography documented the escalation of systolic dysfunction in BYL719-treated post-MI mice with significantly decreased ejection fraction, enlarged left ventricular chamber, and enhanced wall motion abnormality, despite comparable ejection function between groups at baseline (Figure 3.3D).

Because myocardial apoptosis determines the severity of cardiac injury, we evaluated apoptotic rate using TUNEL staining. Although sham-operated hearts displayed negative TUNEL signaling, 1-day post-MI BYL719-treated hearts demonstrated an increase in apoptotic rate compared with controls (Figure 3.4A). Linked to myocardial apoptosis, inflammatory responses were significantly enhanced in post-MI BYL719-treated hearts (Figure 3.4B). In addition, PI3Kα inhibition by BYL719 significantly suppressed post-MI cardiomyocyte hypertrophy without markedly affecting cardiomyocyte size in the sham-operated group (Figure 3.4C). Besides, vascular density was markedly decreased in the peri-infarct area in post-MI BYL719-treated hearts, whilst it was similar between sham-operated groups (Figure 3.4D). Furthermore, phosphorylated protein levels of Akt at Thr308, GSK3β at Ser9, and eNOS at Ser1177 were significantly reduced in PI3Kα-inhibited mice compared with controls at day 7 post-MI without obvious alterations on p110α, phospho-Akt at Ser473, and phospho-ERK levels (Figure 3.4E).

The results indicate that systemic BYL719 administration has detrimental roles in the infarcted heart by enhancing apoptotic cell death, increasing inflammation, suppressing cardiac hypertrophy, and inhibiting vascular repair.
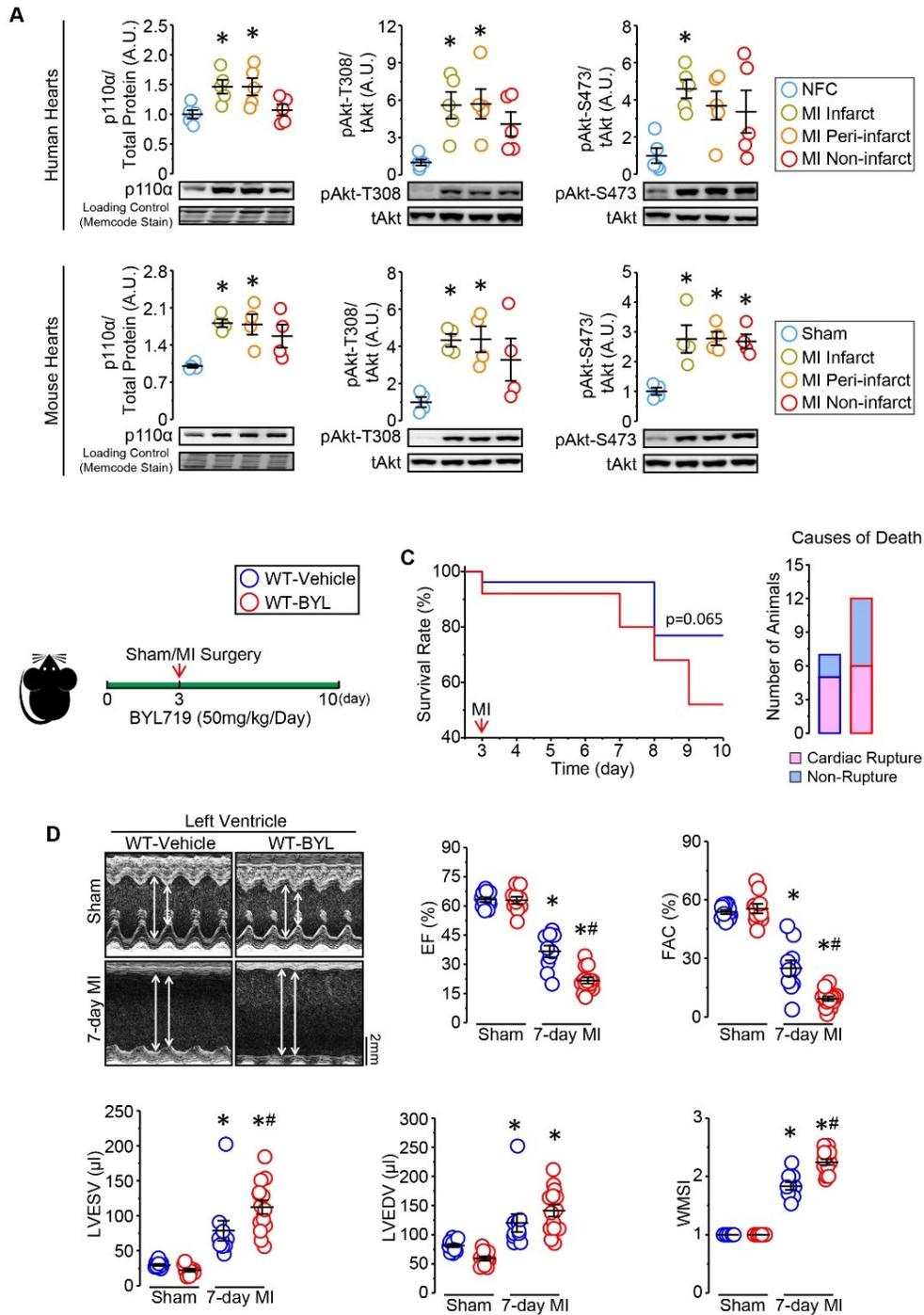
Figure 3. 3. PI3Kα/Akt signaling is increased in post-MI human and mouse hearts; and BYL719 exacerbates cardiac dysfunction after MI. (A) Protein levels of p110α and Akt in control and post-MI hearts. NFC, non-failing control. **(B)** Experimental design testing the influence of MI during BYL719 treatment. **(C)** Kaplan-Meier survival curve and distribution of causes of death in vehicle- and BYL719-treated mice after MI surgery. **(D)** Echocardiographic images showing ventricular morphology and analysis of ejection fraction (EF), fractional area change (FAC), left ventricular end-systolic and end-diastolic volume (LVESV and LVEDV), and wall motion score index (WMSI) in WT-Vehicle and WT-BYL mice. *P<0.05 vs NFC/sham group; #P<0.05 vs p110αFlx.

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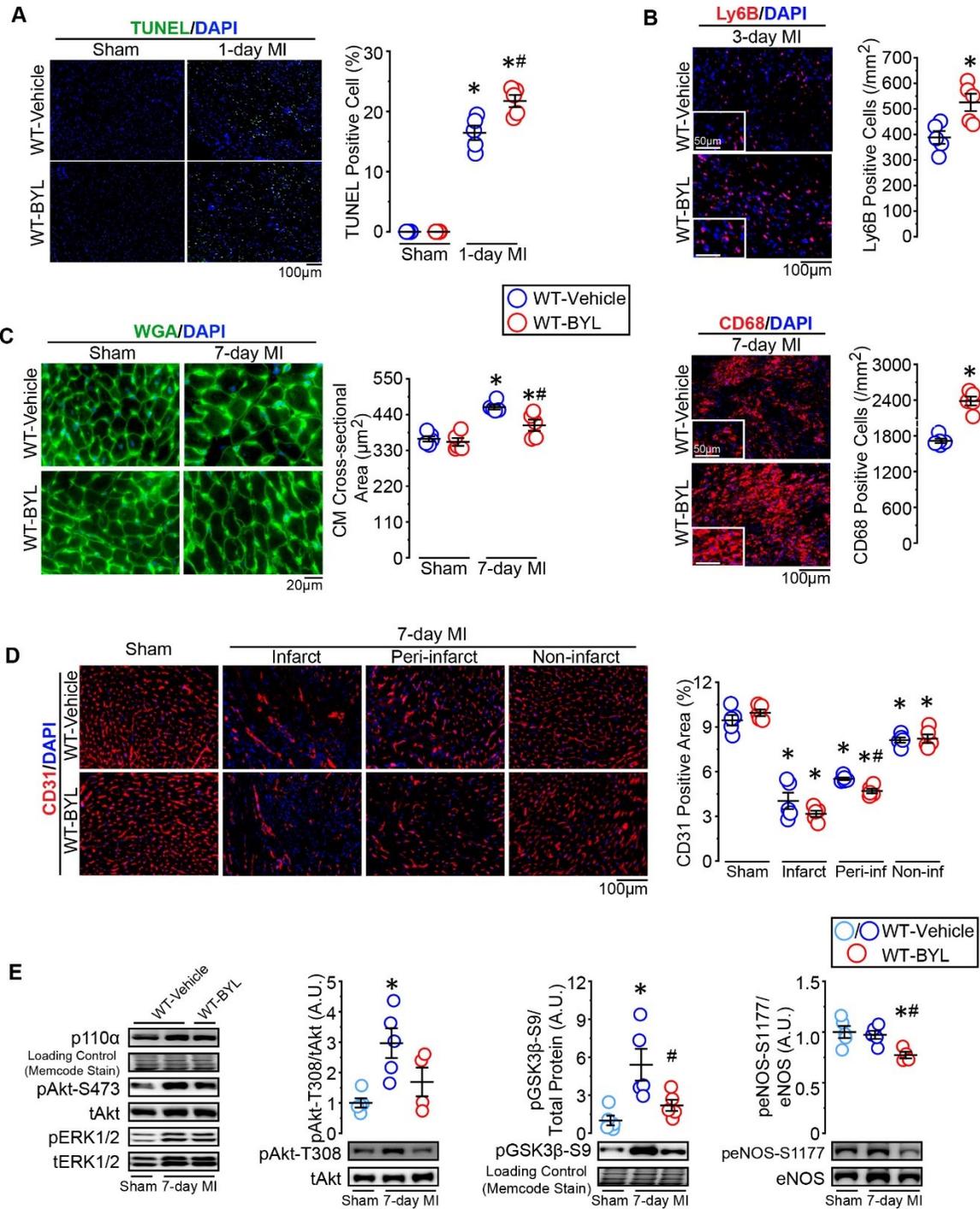
Figure 3. 4. BYL719 leads to adverse ventricular remodeling after MI. (A) Apoptotic cells detected using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and DAPI immunofluorescence staining on 1-day post-MI hearts. **(B)** Immunofluorescence staining for neutrophils (anti-Ly6B) and macrophages (anti-CD68) with DAPI. **(C)** Wheat germ agglutinin (WGA) staining with DAPI to outline cardiomyocyte size. **(D)** Endothelial cell immunofluorescence staining with anti-CD31 to visualize vascular density. **(E)** Western blot analysis of p110α, Akt, ERK1/2, GSK3β, and eNOS in left ventricular lysates. *P<0.05 vs sham group or vs indicated group; #P<0.05 vs p110αFlx.

3.3.3. Endothelial- or Cardiomyocyte-specific PI3Kα Inactivation Aggravated Post-MI Cardiac Dysfunction

In order to delineate the functions of PI3Kα in the heart, and because the two major cell types in the heart are cardiomyocytes and endothelial cells[29], we generated mice expressing kinase-dead PI3Kα specifically in endothelial cells (p110αTie2) or in cardiomyocytes (p110α-αMHC) to unveil the cell-specific roles of PI3Kα in the heart.

First, we examined how endothelial PI3Kα inactivation would affect cardiac function and post-MI cardiac remodeling (Figure 3.5A). Kinase-dead PI3Kα expression in endothelial cells was confirmed by the presence of truncated *Pik3ca* in the lung DNA extraction from p110αTie2 mice (Figure 3.5B); and mice with endothelial PI3Kα inactivation showed comparable body weight, heart weight, left ventricular weight, and the cardiac protein levels of p110α, phospho-Akt, and phospho-eNOS as controls at baseline (Figure 3.5C and 3.5D). Evaluating the cardiac function at baseline, we recorded comparable systolic function in p110αTie2 and control hearts (Figure 3.5E). However, after MI, the deterioration of systolic function was aggravated in 7-day and 4-week post-MI p110αTie2 hearts compared with controls, despite that the post-MI survival rate was comparable (Figure 3.5E-G). Further analysis showed that endothelial PI3Kα inactivation did not affect cardiomyocyte size at baseline and after MI (Figure 3.6A). However, inflammatory cell infiltration was increased in post-MI p110αTie2 hearts compared with controls (Figure 3.6B). Next, as myocardial angiogenesis is critical in infarct healing and decreased vascular density was detected in post-MI BYL719-treated hearts, we performed immunostaining to examine vascular repair processes. We detected an increase in apoptotic endothelial cell ratio at day 1, a decrease in proliferating endothelial cell ratio at day 3, and a reduction in vascular density and functional vasculatures at day 7 post-MI (Figure 3.6C-E). In addition, the expression of phospho-Akt levels was significantly lower in p110αTie2 post-MI hearts than controls (Figure 3.6F). These results indicate that endothelial PI3Kα is required in the infarcted heart to maintain cardiac function by supporting endothelial survival, proliferation, and angiogenesis.
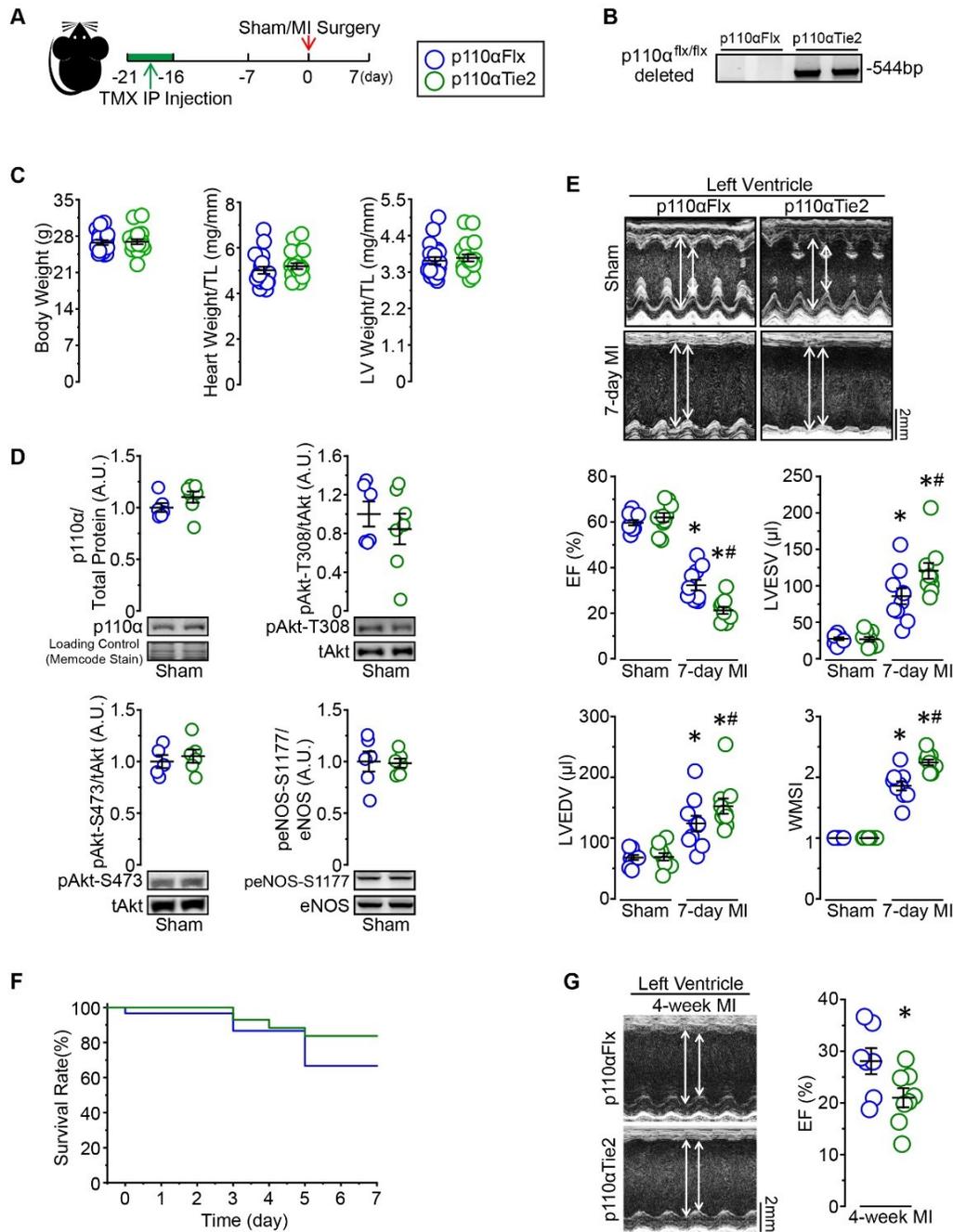
Figure 3. 5. Endothelial PI3Kα inactivation in mice leads to the exacerbation of cardiac function after MI. (A) Experimental design of testing the functions of endothelial PI3Kα using p110αTie2 and control mice. **(B)** PCR analysis amplifying the DNA fragment with partially deleted PIK3ca allele. **(C)** Body weight, heart weight, and left ventricular weight from p110αTie2 and control. **(D)** Western blot analyses accessing p110α, Akt, and eNOS protein levels from left ventricular lysates. **(E)** Echocardiographic images showing left ventricular morphology and functional analysis of ejection fraction (EF), left ventricular end-systolic and end-diastolic volume (LVESV and LVEDV), and wall motion score index (WMSI). **(F)** Kaplan-Meier survival analysis in post-MI p110αTie2 and control mice. **(G)** Echocardiography analysis showing EF on 4-week post-MI p110αTie2 and control mice. *P<0.05 vs sham group or vs indicated group; #P<0.05 vs p110αFlx.
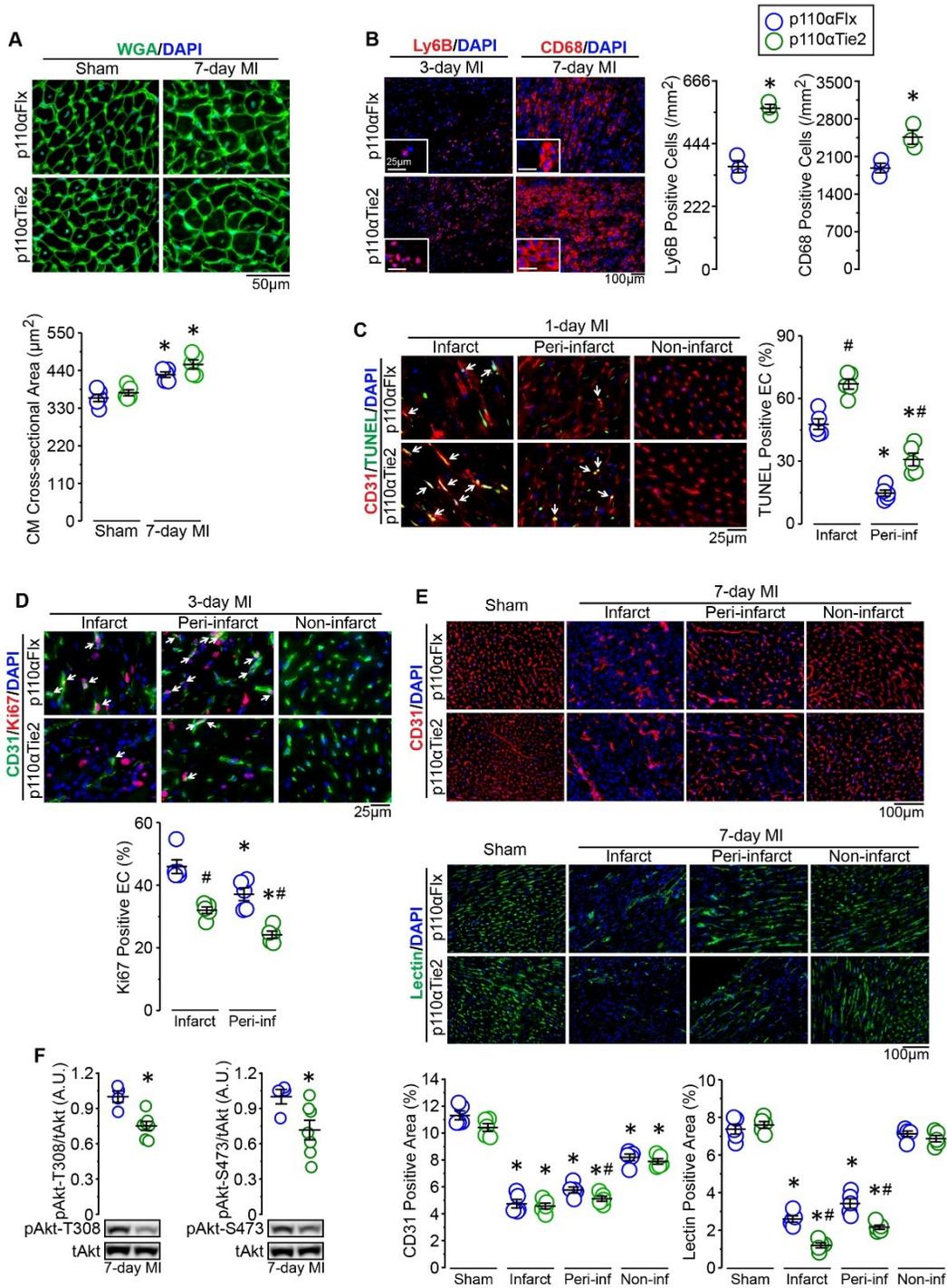
Figure 3. 6. Endothelial PI3Kα is required to maintain cardiac function and angiogenesis after MI. **(A)** Representative immunofluorescence images of wheat germ agglutinin (WGA) staining with DAPI for cardiomyocyte cross-sectional area. **(B)** Immunofluorescence staining for neutrophils (Ly6B) and macrophages (CD68) to examine inflammation. **(C)** Spotting apoptotic endothelial cells using CD31 and TUNEL immunofluorescence staining with DAPI. **(D)** Using CD31 and Ki67 staining to detect proliferating endothelial cells with DAPI. **(E)** CD31 immunofluorescence analysis for vascular density. **(F)** Western blot analysis of Akt level in 7-day post-MI hearts. *P<0.05 vs sham group in A and E, vs indicated group in B and F, and vs infarct group in C and D; #P<0.05 vs p110αFlx.

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Next, we investigated the role of cardiomyocyte PI3Kα in the heart using p110α-αMHC mice (Figure 3.7A). We first confirmed the success of truncated *Pik3ca* expression in p110α-αMHC hearts (Figure 3.7B). We found that p110α-αMHC mice displayed smaller body weight, heart weight, and left ventricular weight than control littermates (Figure 3.7C), which is consistent with the findings from BYL719-treated WT mice. Moreover, p110α-αMHC hearts showed marked reduction in p110α and phospho-GSK3β levels without differences in phospho-Akt level (Figure 3.7D). Furthermore, p110α-αMHC mice displayed a mild reduction in systolic function at baseline (Figure 3.7E). After MI, cardiomyocyte PI3Kα deficiency led to the exacerbation of systolic dysfunction and wall motion abnormality, with significantly increased mortality and cardiac rupture incidence (Figure 3.7E and 3.7F). Evaluating post-MI cardiac remodeling, we found that p110α-αMHC hearts showed no apoptosis at baseline; however, a dramatic increase in apoptotic cells number was detected at day 1 post-MI p110α-αMHC hearts (Figure 3.8A). In addition, post-MI cardiac hypertrophy and vascular density in the non-infarct area was significantly compromised in p110α-αMHC hearts (Figure 3.8B and 3.8C). In addition, Akt phosphorylation was significantly inhibited in p110α-αMHC hearts after myocardial infarction (Figure 3.8D). Therefore, lack of PI3Kα activity not only has detrimental effects on postnatal cardiac development, leading to mild systolic dysfunction, but also severely deteriorates post-MI survival and cardiac dysfunction associated with elevated apoptosis and suppressed cardiac hypertrophy.

Taken together, both endothelial- and cardiomyocyte-PI3Kα inactivated mice share partial similarity with pharmacological PI3Kα inhibition mice under both baseline and post-MI conditions, which indicates that the effects of BYL719 on the non-surgery and infarcted heart are partially due to the inhibition of PI3Kα on endothelial cells and cardiomyocytes. While endothelial PI3Kα is only critical in post-MI vascular repair, cardiomyocyte PI3Kα is essential to maintain normal cardiac function under physiological condition and to support cell survival and hypertrophy when facing MI challenge.
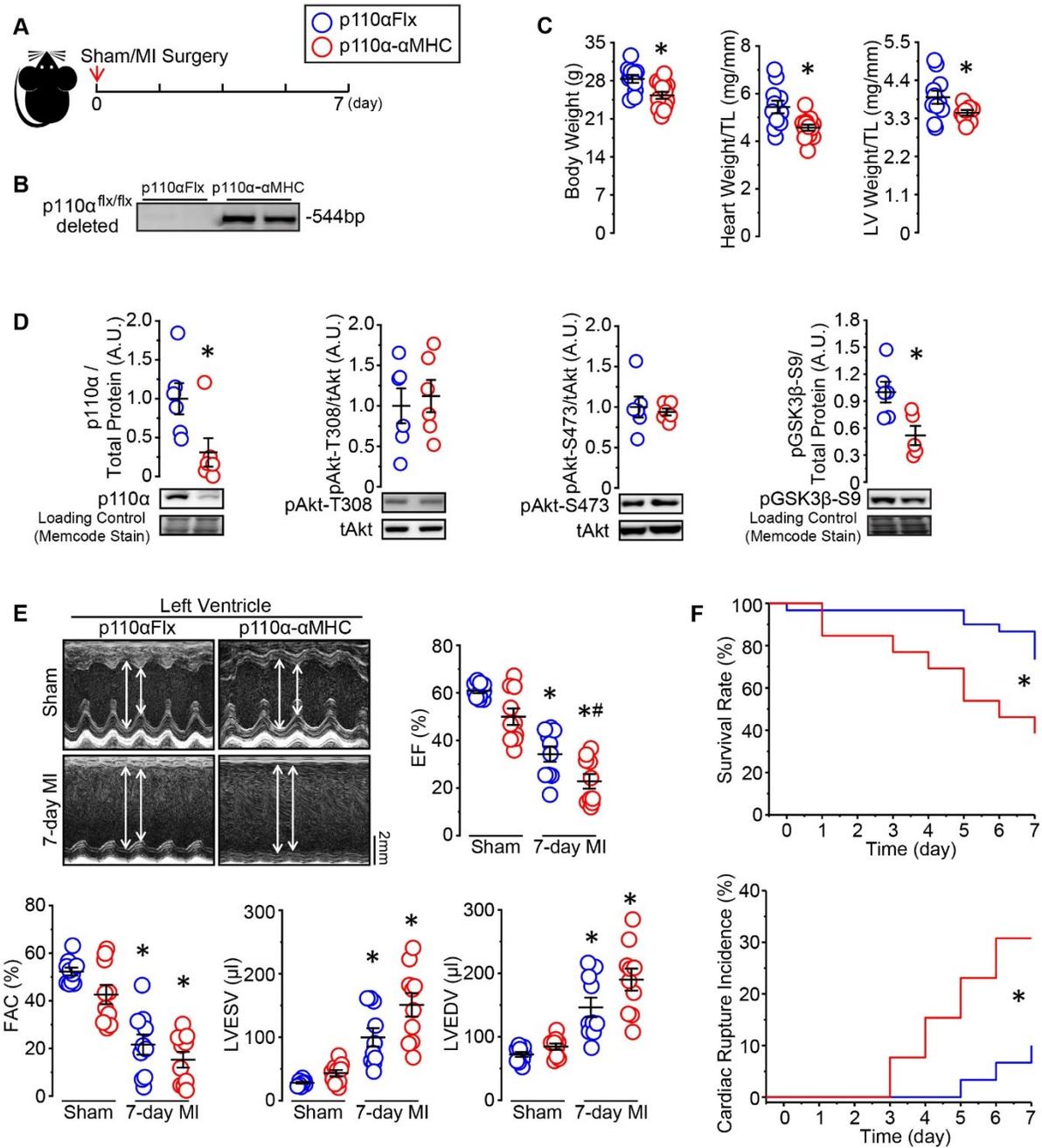
Figure 3. 7. Cardiomyocyte PI3Kα inactivation in mice causes severe systolic dysfunction and increased mortality and cardiac rupture rate. (A) Experimental design on p110α-αMHC and control mice. **(B)** PCR analysis amplifying the DNA fragment with partially deleted PIK3ca allele. **(C)** Body weight, heart weight, and left ventricular weight differences between p110α-αMHC and control mice. **(D)** Western blot analysing p110α, Akt, and GSK3β protein levels from left ventricular lysates. **(E)** Echocardiographic analysis on ejection fraction (EF), fractional area change (FAC), left ventricular end-diastolic volume (LVEDV), and left ventricular end-systolic volume (LVESV). **(F)** Kaplan-Meier survival analysis for survival rate and cardiac rupture incidence. *P<0.05 vs sham group or vs indicated group; #P<0.05 vs p110αFlx.
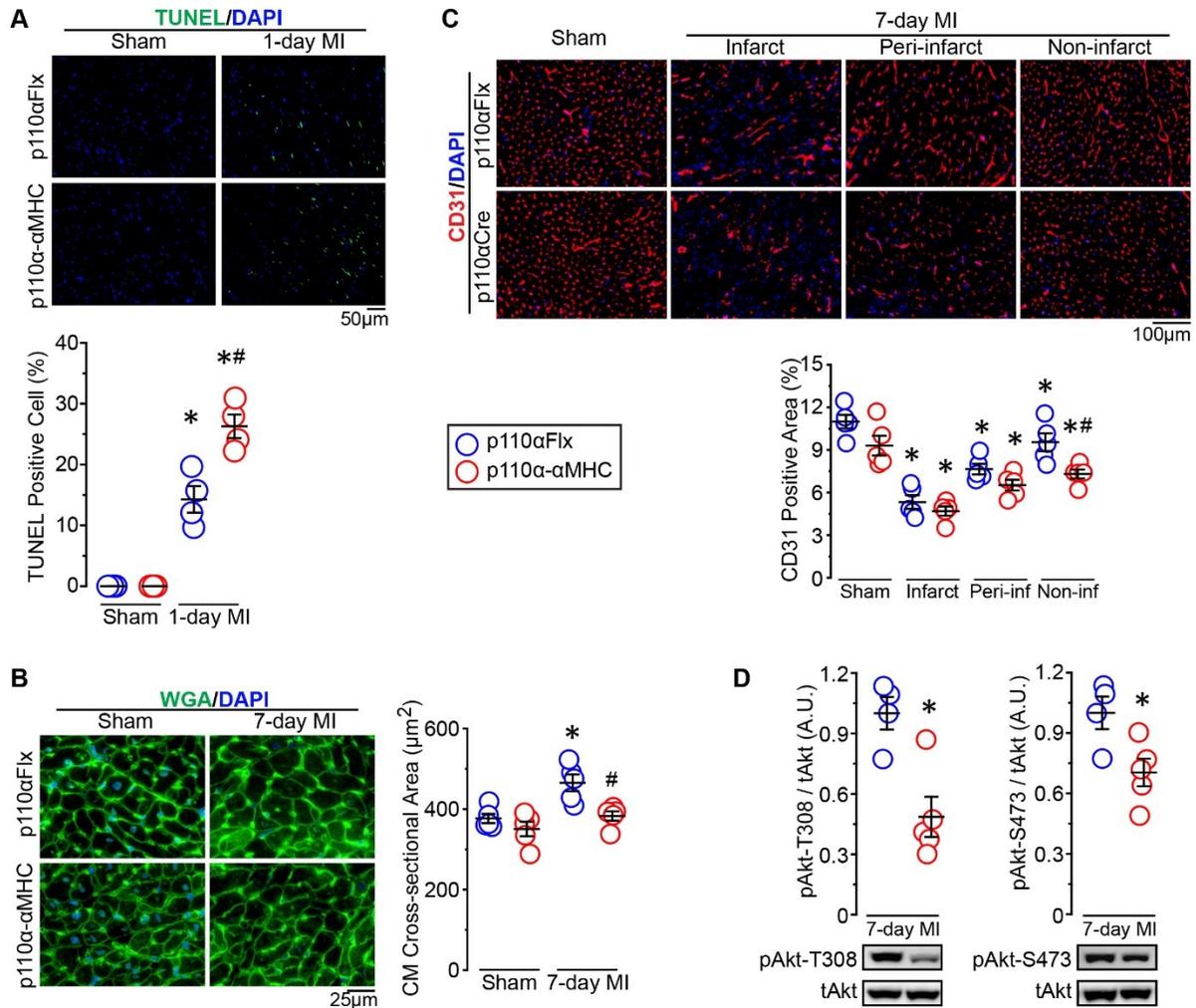
Figure 3. 8. Loss of cardiomyocyte PI3Kα activity is associated with increased cell death, decreased vascular density, and hypertrophy suppression after MI. (A) Accessing apoptosis using TUNEL and DAPI immunofluorescence staining. **(B)** WGA staining outlining cardiomyocyte size. **(C)** Representative immunofluorescence images of CD31 staining with DAPI for vascular density. **(D)** Western blot analysis of Akt level in 7-day post-MI hearts. *$P<0.05$ vs sham group or vs indicated group; #$P<0.05$ vs p110αFlx.

3.3.4. Inhibition of PI3Kα Impairs Angiogenesis

To further evaluate the role of PI3Kα in endothelial cells, we cultured HUVECs, a well-study endothelial cell type, and HCAECs, cardiac-specific endothelial cells, to test the effect of PI3Kα inhibition on endothelial functions and how it affects cardiac angiogenesis. The concentration of 500 nmol/L was chosen as the highest concentration tested in the experiments because it is approximately the lowest concentration in the plasma of patients with continuous BYL719 treatment, making this concentration more clinically relevant[206]. As PI3Kα primarily responses to RTKs such as vascular endothelial growth factor receptors (VEGFRs) on

endothelial cells, we first tested the effect of BYL719 inhibition on VEGF-induced PI3K/Akt signaling. Responding to an increase in BYL719 concentration, Akt phosphorylation was progressively decreased; and at the concentration of 500 nmol/L, BYL719 completely blocked VEGF-stimulated Akt phosphorylation on HUVECs (Figure 3.9A). However, VEGF-induced eNOS phosphorylation was suppressed only at high BYL719 concentration-500 nmol/L (Figure 3.9A). In the HCAECs, BYL719 also inhibited Akt activation following VEGF stimulation; but HCAECs showed higher sensitivity to BYL719 compared to HUVECs as both 100 nmol/L and 500 nmol/L completely brought VEGF-induced Akt activation back to baseline, with lower inhibitory effect on eNOS phosphorylation (Figure 3.9B). Nevertheless, these results suggest that BYL719 specifically inhibits VEGF-stimulated Akt signaling in HUVECs and HCAECs, with partially inhibitory effects on eNOS phosphorylation. Next, testing the effects of BYL719 on cultured cells with complete angiogenic supplements, we showed that, over 500 nmol/L concentration, BYL719 suppressed Akt activity without significantly affecting phospho-eNOS and p110α protein levels on HUVECs, while both 100 nmol/L and 500 nmol/L BYL719 similarly inhibited Akt/eNOS signaling on HCAECs (Figure 3.9C and 3.9D). Altogether, these results indicate that BYL719 inhibits Akt/eNOS signaling in HUVECs and HCAECs, with HCAECs being more susceptible to PI3Kα inhibition.

Next, we examined the effects of PI3Kα inhibition on endothelial cell survival, proliferation, and angiogenesis. Treated with 500 nmol/L BYL719, the majority of HUVECs underwent cell death through apoptosis, not necrosis; moreover, PI3Kα inhibition with BYL719 decreased the number of viable cells, increased apoptosis rate, and reduced proliferation rate (Figure 3.10A and 3.10B). Furthermore, Angiogenic sprouting was significantly inhibited with BYL719 treatment compared with control (Figure 3.10C). These results indicate that PI3Kα activity is required for HUVEC survival, proliferation, and angiogenic sprouting upon angiogenic stimulation. Then, we tested how BYL719 affects angiogenic responses in cardiac endothelial cells. Survival analyses showed that BYL719 treatment decreased cell viability, increased apoptosis, and decreased cell proliferation as HUVECs (Figure 3.10D and 3.10F). Further angiogenic assay depicted the inhibitory effect of BYL719 on the development of angiogenic sprouts and the length of sprouts (Figure 3.10F).

Taken together, our results suggest that BYL719 is specific and potent PI3Kα inhibitor on HUVECs and HCAECs, and endothelial cells require PI3Kα to maintain Akt/eNOS signaling, cell survival, proliferation, and angiogenesis.
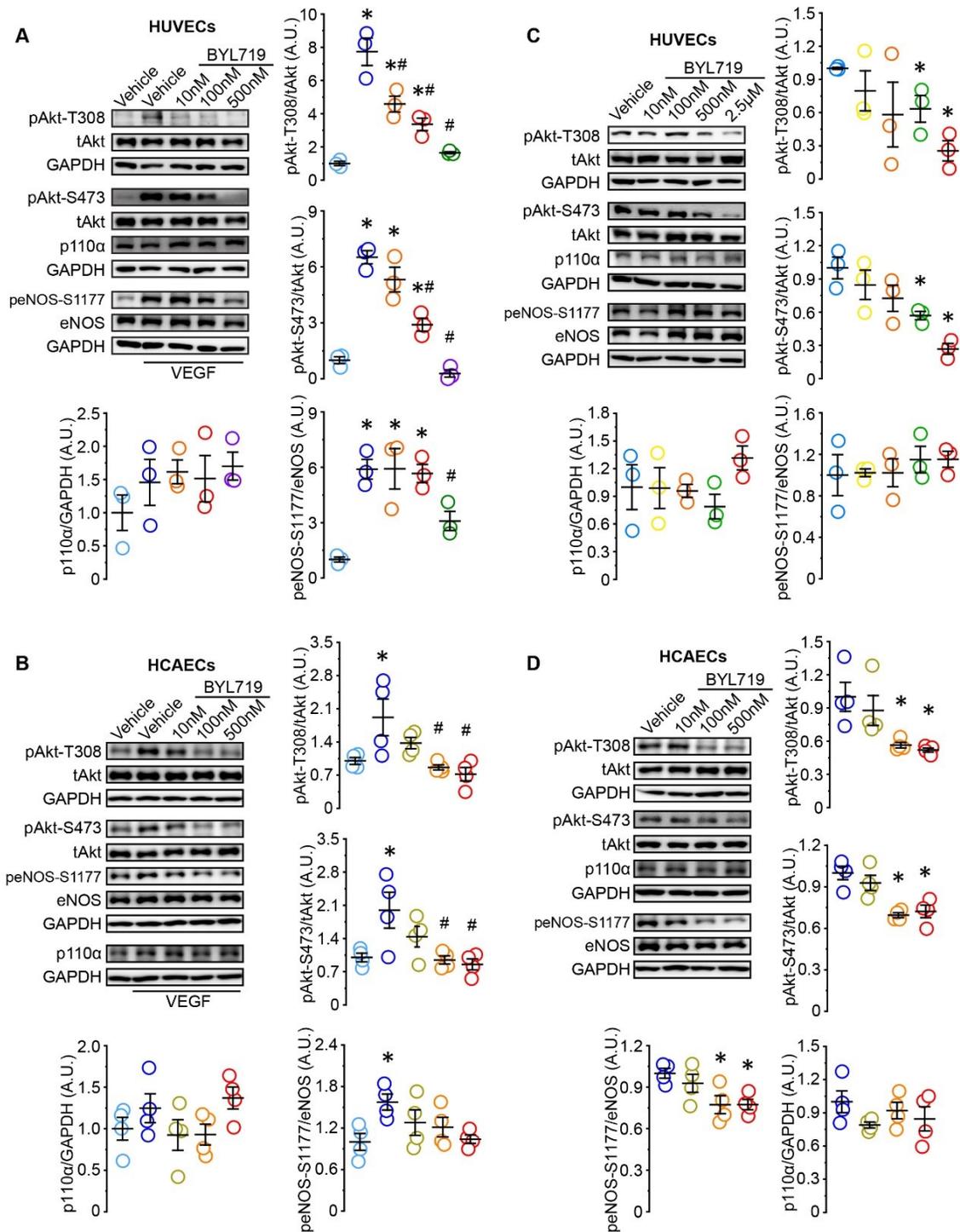
Figure 3. 9. Endothelial cells require PI3Kα to maintain Akt/eNOS signaling. (A) Effects of BYL719 on vascular endothelial growth factor (VEGF)-induced Akt/eNOS activation testing by Western blot on HUVECs. **(B)** Effects of BYL719 on VEGF-induced Akt/eNOS activation testing by Western blot on HCAECs. **(C)** Impact of BYL719 on Akt/eNOS signaling in cultured HUVECs detecting by Western blot. **(D)** Impact of BYL719 on Akt/eNOS signaling in cultured HCAECs detecting by Western blot. *P<0.05 vs vehicle without VEGF in A and B and vs vehicle group in C and D; #P<0.05 vs vehicle with VEGF.
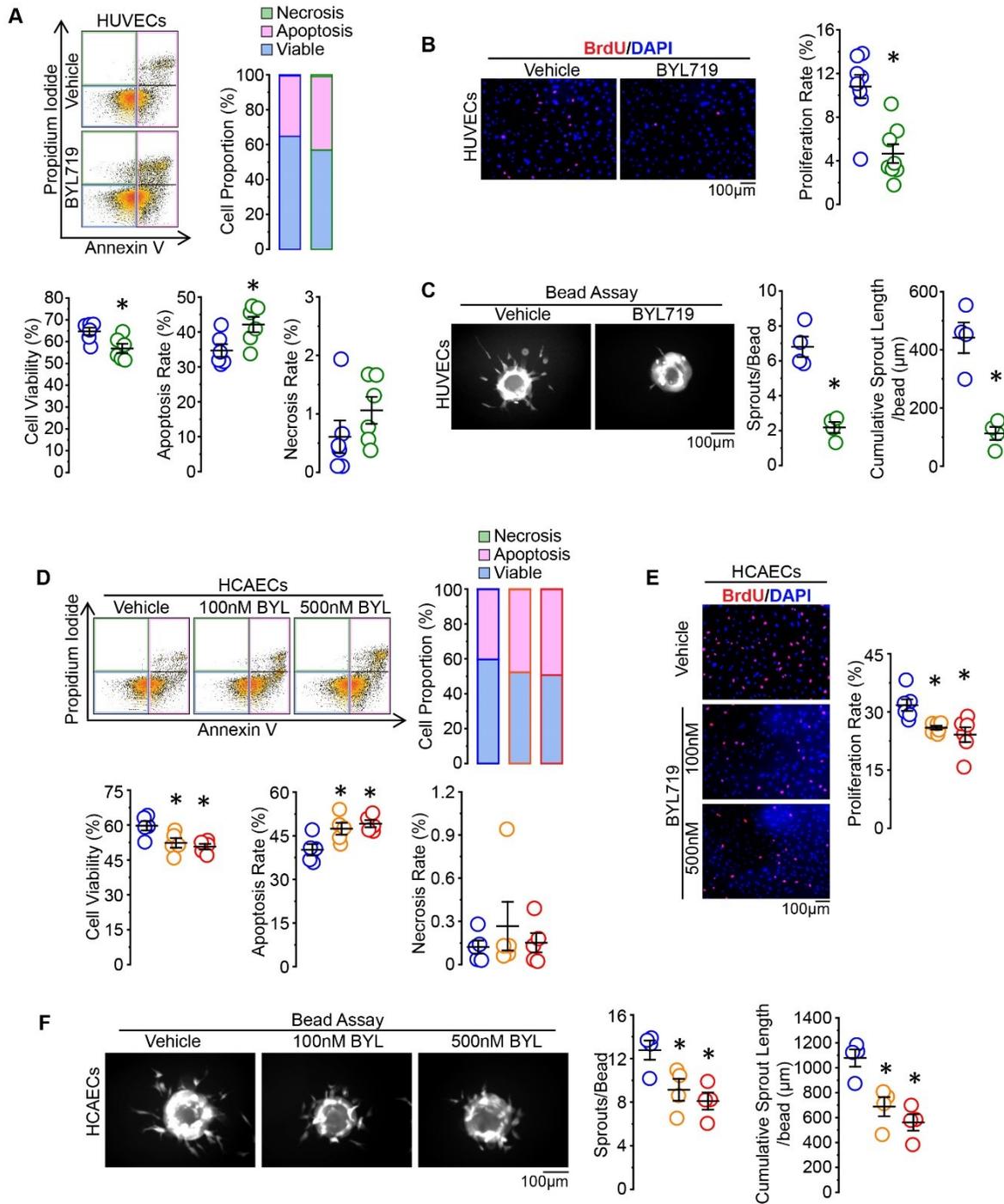
Figure 3. 10. PI3Kα activity is dispensable in endothelial cell survival, proliferation, and angiogenesis. (A) Flow cytometry images and quantification data for HUVEC survival staining with annexin V and propidium iodide. **(B)** Accessing cell proliferation rate using BrdU and DAPI immunofluorescence staining on HUVEC treated with vehicle or BYL719. **(C)** Bead assay showing endothelial sprouts in HUVECs with and without BYL719. **(D)** Flow cytometry images and quantification data for cell survival staining with annexin V and propidium iodide. **(E)** Accessing cell proliferation rate using BrdU and DAPI immunofluorescence staining on HCAECs. **(F)** Bead assay testing the effect of BYL719 on HCAECs. *P<0.05 vs indicated group in A-C and vs vehicle group in D-F.

3.3.5. Cardiomyocyte PI3Kα Protects Hypoxia-induced Cell Death

To elucidate the roles of PI3Kα in cardiomyocyte survival, adult mouse cardiomyocytes were isolated from WT mice. BYL719 had no effect on Akt activity under normoxic condition; however, BYL719 inhibited hypoxia-related Akt activation in a dose-dependent manner with 500 nmol/L markedly blocking Akt and GSK3β phosphorylation (Figure 3.11A). And this effect was not caused by cross-inhibition of PI3Kγ by BYL719 because additional PI3Kγ inhibition with AS252424 had let to further decrease in Akt phosphorylation without effects on GSK3β phosphorylation (Figure 3.11B). Thus, PI3Kα is required in hypoxia triggered Akt/GSK3β signaling.
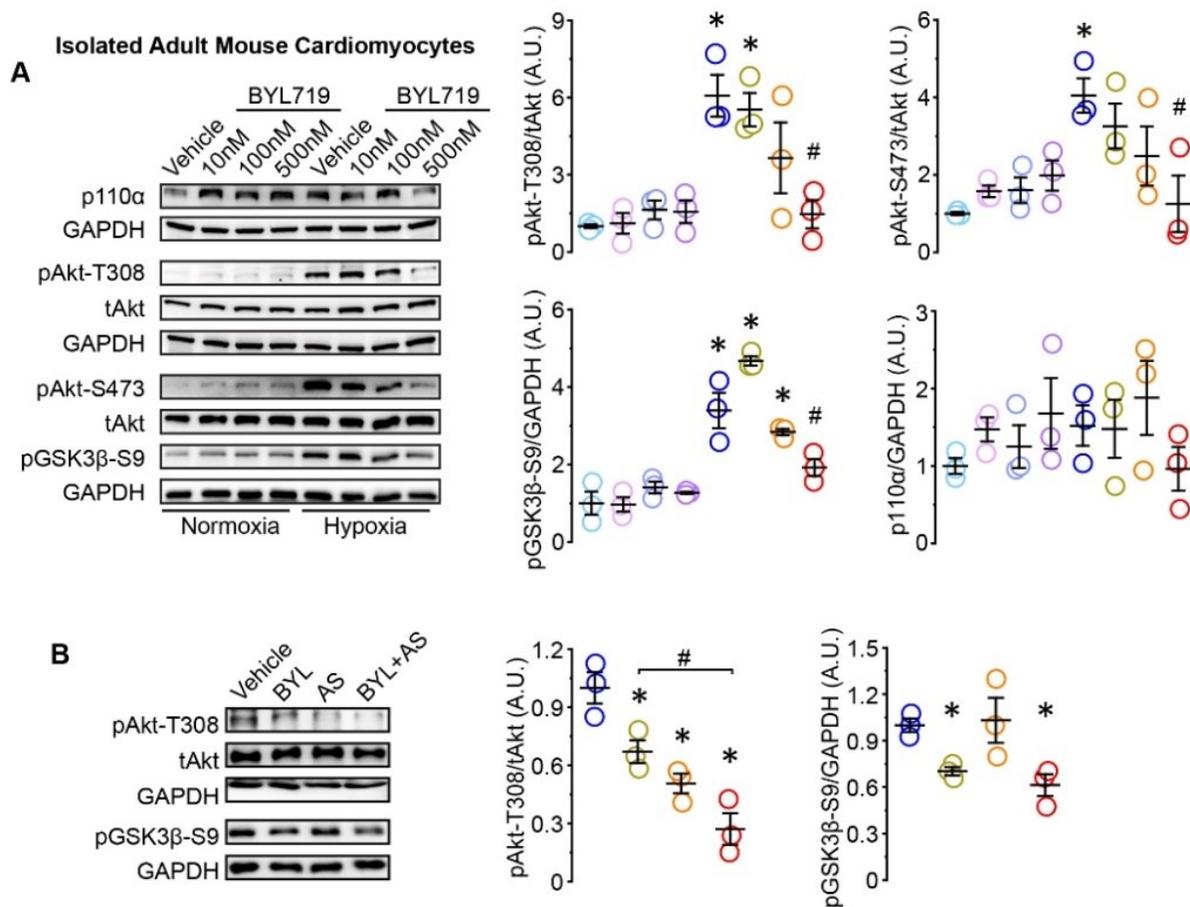
Figure 3. 11. Hypoxia induced Akt/GSK3β signaling is through PI3Kα. (A) Western blot analyses on lysates from cardiomyocytes cultured under normoxic and hypoxic conditions. **(B)** Western blot analyses on isolated cardiomyocyte lysates with PI3Kα inhibitor-BYL719 and PI3Kγ inhibitor-AS252424 (AS). *P<0.05 vs normoxic group in A and vs vehicle group in B; #P<0.05 vs hypoxic vehicle group in A and vs indicated group in B.

Next, we cultured isolated cardiomyocytes under hypoxia with cyclic mechanical stretching system to simulate *in vivo* condition. With 3-hour mechanical stretching, the morphologic study revealed a comparable number of rod-shaped cardiomyocytes with and without BYL719; however, cardiomyocyte apoptosis rate was increased with BYL719 treatment (Figure 3.12A and 3.12B). Our previous work has identified the role of PI3Kα/PIP3 in maintaining F-actin density[152]. Here, we showed that hypoxia increased F-/G-actin ratio in stretched cardiomyocytes, and PI3Kα inhibition has significantly decreased F-/G-actin ratio under hypoxic condition (Figure 3.12C). Overall, these results indicate that PI3Kα is necessary to induce hypoxia associated Akt activation, cell survival, and cytoskeleton remodeling.
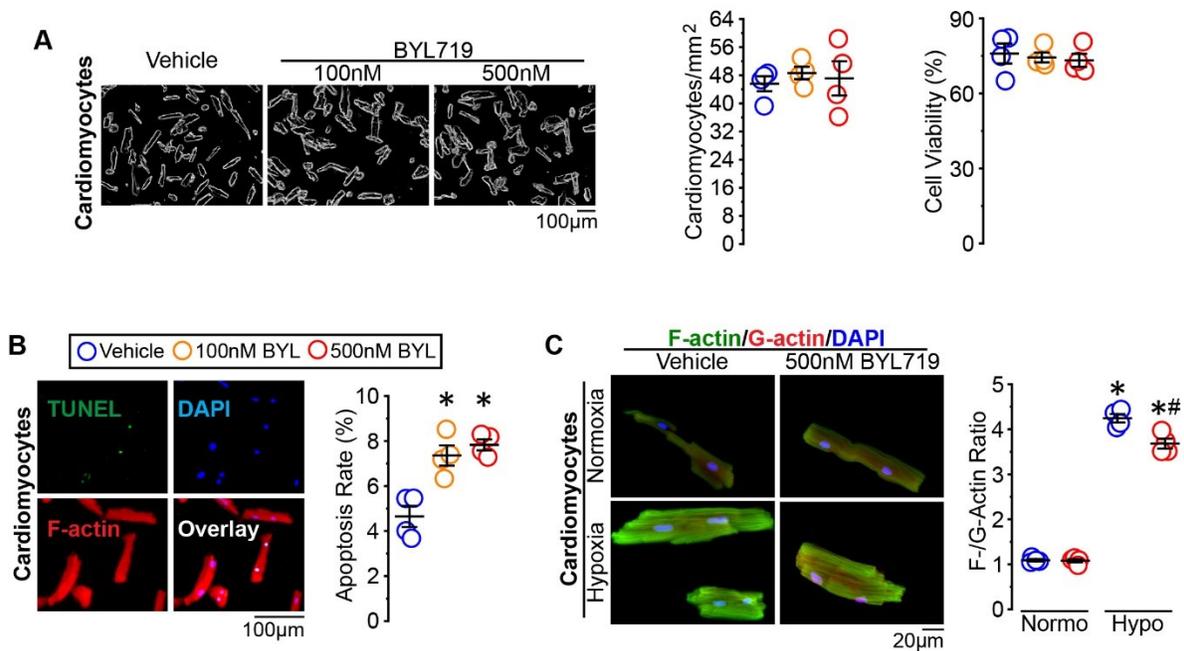
Figure 3. 12. PI3Kα inhibition leads to an increase in hypoxia induced apoptosis and a reduction in hypoxia triggered cytoskeleton remodeling. (A) Representative images and analyses of cell viability from isolated cardiomyocyte under hypoxic condition with cyclic mechanical stretch. **(B)** Representative immunofluorescence images of TUNEL staining with F-actin and DAPI showing apoptotic cells and quantification of apoptosis rate. **(C)** Representative images depicting F-actin and G-actin in stretched cardiomyocytes under normoxic or hypoxic condition. *P<0.05 vs vehicle in B and vs normoxic group in C; #P<0.05 vs vehicle group.

3.4. Discussion

The coexistence of cardiovascular disease (CVD) and cancer, the two leading causes of death worldwide, have received increasing awareness in recent years[242,243]. Evidence has linked cancer with a higher incidence of ischemic heart disease, and vice versa[244,245], which has been attributed to the shared risk factors, the consequence of one disease-focused treatment, and

the systematically molecular alterations such as chronic inflammation and oxidative stress[246-248]. Thus, optimization of both IHD and cancer care is critical. In this study, we revealed that PI3Kα inhibitor-BYL719, the novel anti-cancer drug, has detrimental effects on cardiac health and post-MI cardiac repair, unveiling the central role of PI3Kα in wound healing after MI. We further specified that both endothelial and cardiomyocyte PI3Kα are crucial in post-MI cardiac healing (Figure 3.13). While endothelial PI3Kα critically participates in angiogenic processes to maintain vascular distribution in the ischemic heart, cardiomyocyte PI3Kα partially regulates cardiac contractility under physiological condition, and most importantly, prevents cardiomyocyte apoptosis, supports cardiac hypertrophy, and maintains F-/G-actin ratio under ischemia. Therefore, the augmentation of PI3Kα activity may assist the preservation of cardiac function in the ischemic heart, whilst anti-cancer therapy with PI3Kα inhibitors may raise the potential of cardiac toxicity to the heart, especially to the ischemic heart.
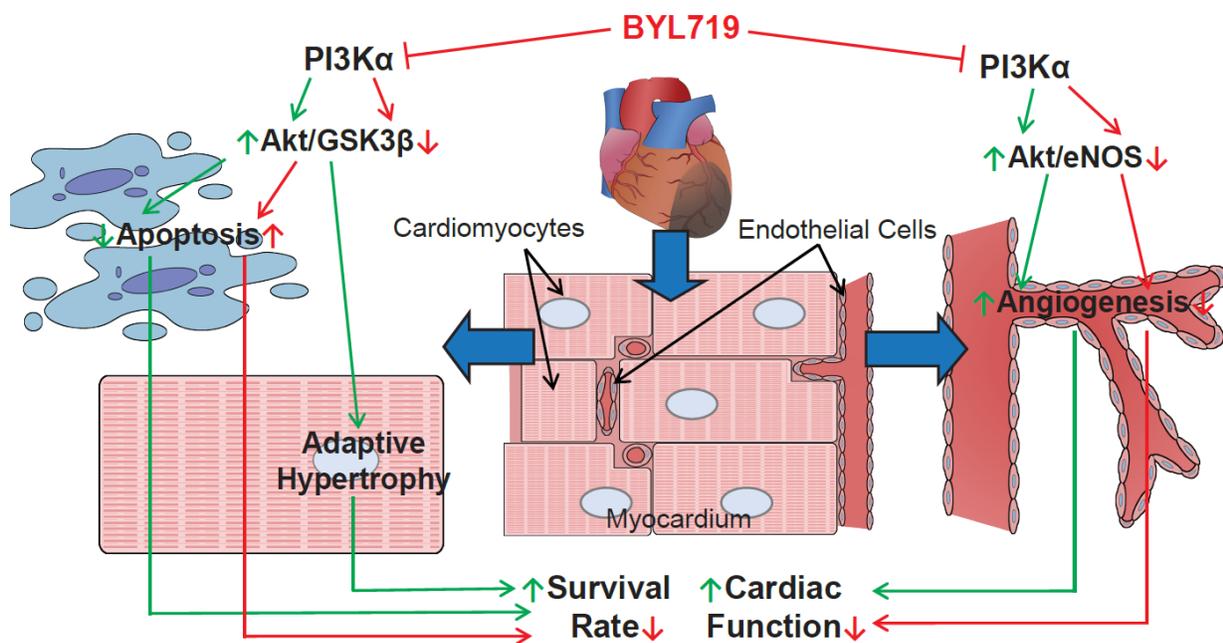
Figure 3. 13. PI3Kα is critical to maintain cardiomyocyte survival and hypertrophy and support angiogenesis after MI.

Activation of the PI3Kα/Akt signaling pathway contributes significantly to cancer development and progression as PI3Kα activity is frequently altered in a variety of the common human tumors[228]. With this notion, PI3Kα-specific inhibitors, such as alpelisib (BYL719) and serabelisib, have been developed and have achieved initial success on advanced solid tumor treatment especially when combined with standard anti-cancer therapy[206,249-251]. However, it is

known that PI3Kα inhibitors also generate systemic effects. Besides confirming that PI3Kα inhibition causes gradual weight loss and transient hyperglycemia as others[203,215], our study also revealed that BYL719 led to lean mass reduction and fat mass increase which are associated with poor outcomes in CVD[252]. Moreover, despite that the major toxic effects of PI3Kα inhibitors reported in clinical trials are hyperglycemia, cutaneous reactions, and gastrointestinal discomforts[253], our pre-clinical studies have also indicated the potential of PI3Kα inhibitors-related cardiotoxicity. Previously, our team has demonstrated that long-term BYL719 treatment has led to heart weight reduction, cardiomyocyte size reduction, and QTc interval prolongation[212,215], we here showed partly similar results in mice with 10-day BYL719 administration; and, most importantly, we detected compromised left ventricle global longitudinal strain in these mice using strain analysis, indicating the existence of subtle cardiac dysfunction under BYL719 treatment. Both prolonged QTc interval and lower GLS have been evidently associated with higher risk of cardiovascular morbidity and mortality[254,255]. Thus, the combined results suggest that the systemic and cardiac-specific effects of PI3Kα inhibitors predispose to higher cardiovascular risks.

Accordingly, we studied genetically modified mice with endothelial- or cardiomyocyte-PI3Kα inactivation to specifically focus on the effects of PI3Kα activity in the cardiac tissue. Lining the blood vessels in a highly vascularized cardiac tissue[29], cardiac endothelial cells are the gatekeeper between blood and cardiac parenchymal, making them susceptible to the administration of PI3Kα inhibitors. We showed that endothelial PI3Kα is not required to maintain vascular density and cardiac function under physiologic conditions, which might be attributed to the quiescent and non-proliferative status of the endothelial cells under physiologic condition. Unlike endothelial PI3Kα showing no obvious influence on cardiac function in adult mice, cardiomyocyte PI3Kα is required to maintain normal cardiac function. Previous studies on cardiac-specific constitutive-active or dominant-negative PI3Kα mice have demonstrated normal systolic function with a bigger or smaller heart[188]. Our results on genetic ablation of cardiomyocyte PI3Kα demonstrated a decrease in heart weight as well. However, a mild decrease in cardiac function was also suggested, which is consistent with previous findings on mice with inducible PI3Kα gene deletion where the decreased cardiac function has been attributed to the reduction of L-type calcium channels[180]. Additionally, we spotted a decrease in GSK3β inactivation with decreased PI3Kα level, despite no obvious changes on Akt phosphorylation or evidence of apoptosis, which suggests that compensatory processes, potentially via other PI3K isoforms, might take place to maintain Akt activity at baseline, while GSK3β phosphorylation is heavily dependent on PI3Kα. This was confirmed in isolated

cardiomyocyte culture experiments showing PI3Kα, not PI3Kγ, was responsible for hypoxia induced GSK3β phosphorylation. Overall, a minimum requirement of cardiomyocyte PI3Kα activity might be required to maintain proper cardiomyocyte size and cardiac function, but lack of PI3Kα does not induce cell death without pathologic stimulus despite that one of the most important roles of PI3K/Akt pathway is controlling cell survival[92].

However, it is a different story under MI challenge. In this study, we demonstrated that PI3Kα and Akt phosphorylation were significantly upregulated after MI, suggesting the participation of PI3Kα/Akt signaling in ventricular remodeling. Experiments on BYL719-treated mice revealed that PI3Kα inhibition caused a slight increase in mortality, a marked decrease in cardiac function, and adverse ventricular remodeling despite no overt cardiac dysfunction on sham-operated mice. No significant difference on the cause of post-MI death between BYL719 and controls was depicted, regardless of the predicted effects of drug-induced QTc prolongation on sudden cardiac death in IHD patients[256]. This result suggests that other aspects of adverse ventricular remodeling, not electrophysiological remodeling, might be the culprits of the short-term adverse outcomes in this animal model.

Post-MI cardiac remodeling is a series of complex and highly orchestrated events involving cardiomyocyte death and growth, inflammation, vascular repair, and fibrosis, which determines patient outcomes[257]. While balanced inflammatory and fibrotic responses are required in post-MI cardiac repair to maintain cardiac integrity and function, prevention of cardiac apoptosis and maintenance of cardiac vasculatures provide beneficial effects to the ischemic heart[15,237,258]. Ischemic preconditioning, postconditioning, and many compounds have been demonstrated to protect against myocardial apoptosis through PI3K/Akt pathway, making PI3K/Akt the central regulator of the anti-apoptotic effect[164,259-261]. Moreover, PI3K/Akt signaling have established an essential role in angiogenesis for decades. *In vitro* cell cultures have demonstrated the dispensable roles of PI3K/Akt in endothelial cell survival, migration, elongation, and angiogenesis[170,172,262,263]. Besides, *in vivo* use of pan-PI3K inhibitor has exacerbated the cardiac dysfunction and reduced angiogenesis in post-MI swine model[163]. Here, we discerned the PI3Kα isoform of the PI3Ks is of great importance in regulating myocardial apoptosis and cardiac angiogenesis following MI. And to our best knowledge, this is the first paper to investigate the implications of pharmacological PI3Kα inhibitor on cardiac health and after myocardial ischemic injury. We demonstrated that inhibition of PI3Kα isoform leads to increased mortality incidence, exacerbated cardiac dysfunction, enhanced apoptotic cell death, elevated inflammation, decreased cardiomyocyte hypertrophy, and diminished vascular density after MI. This model shared some similarity with genetically modified animal

models with cardiomyocyte- or endothelial-specific PI3Kα inactivation. While lack of cardiomyocyte PI3Kα activity promotes post-MI cardiac rupture and systolic dysfunction associated with enhanced myocardial apoptosis and suppressed hypertrophy, endothelial PI3Kα primarily regulates cardiac function through angiogenic responses. In addition, animal models with BYL719 and in vitro experiment on endothelial cells and cardiomyocytes confirm that PI3Kα inhibitor suppresses Akt/GSK3β/eNOS phosphorylation, leading to GSK3β overactivation and eNOS inhibition. Previous research has evidenced that deficiency of cardiac GSK3β and overexpression of eNOS have protected against post-MI remodeling by regulating cardiac hypertrophy and capillary density[165,264]. Thus, these results suggest that systemic administration of PI3Kα inhibitor impacts post-MI cardiac remodeling and repair partially through inhibiting cardiomyocyte and endothelial PI3Kα activity.

Under hypoxic condition, cardiomyocytes upregulate PI3K/Akt/GSK3β signaling pathway to counteract apoptosis through regulating apoptosis-related proteins such as Bad, modifying mitochondrial function through mitochondrial translocation, and influencing nuclear activity[265-268]. We documented that the increase in PI3K/Akt signaling is blocked by PI3Kα inhibition, which is associated with increased apoptotic rate. In conjunction with other studies, cardiac PI3Kα positively regulates cardiac remodeling following dilated cardiomyopathy, pressure overload-induced heart failure, and MI-associated heart failure[152,184,188,238]. In contrast to cardiomyocytes lacking regenerative capability, endothelial cells are capable of transiting from quiescent state to proliferative and angiogenic state under pathologic circumstances, especially under MI. We agreed with others that PI3Kα is preferentially activated downstream RTKs, such as VEGF receptors[222]. And previous studies have highlighted the regulatory roles of PI3Kα in endothelial adherens junctions and endothelial cell migration[189,192]. In this study, we showed that angiogenic factors-induced endothelial survival, proliferation, and angiogenesis are significantly compromised under PI3Kα inhibition in cardiac-specific endothelial cells, which is associated with Akt/eNOS signaling suppression; moreover, these findings are consistent with in vivo animal models showing PI3Kα suppression remarkably inhibits cardiac repair processes in the ischemic heart, causing the decrease in vascular density and deterioration of cardiac function. Hence, PI3Kα exerts differentially functions in cardiomyocytes and endothelial cells in the ischemic heart. It regulates cardiomyocyte apoptosis and endothelial angiogenesis to influence cardiac repair and remodeling.

From a clinical standpoint, our results are significant because (i) they suggest the therapeutic potential of manipulating cardiac PI3Kα to improve post-MI cardiac repair by supporting cell survival and angiogenesis; (ii) they address the idea that the use of PI3Kα

inhibitor is detrimental to the heart, especially to the ischemic heart; and these effects are partially attributed to the effects of PI3Kα inhibitor on cardiomyocytes and endothelial cells as cardiomyocyte- or endothelial-specific inactivation of PI3Kα displays similar findings in the healthy and ischemic heart. There is plenty of clinical research indicating the anti-cancer effects of PI3Kα inhibitor, but limited literature demonstrating the long-term effect of PI3Kα inhibitor on the heart and CVD morbidity and mortality. Thus, this study highlights the importance of monitoring cardiac function while taking PI3Kα inhibitor, being cautious in patients with high CVD risk, and promoting CVD risk factor modification in patients. However, further exploration of cardiac health after PI3Kα inhibitor withdrawal and the detailed mechanisms into the intracellular signaling of PI3Kα could be profoundly beneficial to minimize the adverse effects of PI3Kα inhibitors on the heart, particularly in patients experiencing MI during PI3Kα inhibitor treatment.

Chapter 4

Endothelial and Cardiomyocyte PI3Kβ Divergently Regulate Cardiac Remodeling in Response to Ischemic Injury

Endothelial and Cardiomyocyte PI3Kβ Divergently Regulate Cardiac Remodeling in Response to Ischemic Injury

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4.1. Introduction

Ischemic heart disease (IHD) has a high risk of developing heart failure (HF) with a high morbidity and mortality burden worldwide[269]. Given the high metabolic demands of the heart, disruption of coronary blood flow leads to loss of cardiomyocyte followed by scar formation, both of which are characteristic changes of myocardial infarction (MI) and ischemia-reperfusion (IR) injury[270,271]. Hence, improving vascular supply by proangiogenic therapy and preventing cardiomyocyte death can protect the ischemic myocardium. During angiogenesis, various angiogenic signals, such as vascular endothelial growth factor (VEGF), activate quiescent endothelial cells, which proliferate and differentiate to form microvascular sprouts and ultimately neovessels, rescuing peri-infarct cardiomyocytes and preventing the transition to HF[272]. Meanwhile, necrosis, apoptosis, and autophagy are major contributors to cardiomyocyte death in ischemic hearts, and the extent of cardiomyocyte loss determines infarct size, cardiac function, and patient outcomes[15].

Phosphoinositide 3-kinase (PI3K) signaling pathways are central determinants of cellular response to injury and play a critical role in promoting angiogenesis and cell survival[273]. Members of the class I_A PI3Ks are composed of a p110 catalytic subunit (p110α, β, and δ) and a regulatory subunit. Activation of insulin- or growth factor-receptor complexes stimulate PI3Ks which promote the production of phosphatidylinositol-3,4,5-trisphosphate (PIP3) and plasma membrane recruitment of PIP3-binding proteins, regulating various cellular responses. VEGF and its receptors signal through PI3Kα/Akt/endothelial nitric oxide synthase (eNOS) pathway in endothelial cells, controlling cell survival, migration, vascular permeability, and vessel sprouting[171,189,190]. In cardiomyocytes, PI3Kα/Akt signaling regulates ischemia-driven cardiomyocyte apoptosis, hypertrophy, and contractile function[162,238].

As a member of the class I_A PI3Ks, PI3Kβ isoform is ubiquitously expressed and was originally considered being functionally redundant because PI3Kβ functions are similar to other PI3Ks (PI3Kα and PI3Kγ)[94,195]. In endothelial cells, PI3Kβ activity is lower than PI3Kα, and mice harboring inactivated-PI3Kβ endothelial cells display normal vasculature, suggesting a dispensable role of PI3Kβ during embryonic vascular development[189]. However, the role of PI3Kβ in the response to injury has not been examined. Using genetic murine models, we examined the cell-specific function of PI3Kβ in endothelial cells versus cardiomyocytes in response to myocardial ischemic injury. Here we demonstrate that inhibition of endothelial PI3Kβ protects the ischemic heart by promoting the PI3Kα/Akt/eNOS signaling pathway and angiogenesis, while inhibition of PI3Kβ activity in cardiomyocytes exacerbates the adverse cardiac remodeling post-MI by sensitizing cardiomyocytes to ischemia-triggered cell death.

4.2. Methods

Detailed methods used in the present study are available in **Chapter 2 Materials and Methods**.

4.2.1. Animal Models and Human Explanted Hearts

Mice with PI3Kβ-inactivation under the control of Tie2 promoter-driven conditionally-active Cre recombinase (p110βTie2) or α-MHC promoter-controlled constitutively-active Cre (p110β-αMHC) were generated as described[94,214]. Homozygous littermates p110β$^{flox/flox}$ (p110βFlx) mice were used as control. Tamoxifen (Sigma-Aldrich) was given to 10-week-old p110βTie2 and littermate controls to activate Cre in endothelial cells. Cre deletes exons 21 and 22 from *Pik3cb* (gene encoding p110β), producing a truncated PI3Kβ which lacks catalytic activity. Animal experiments were conducted in accordance with the Canadian Council for Animal Care guidelines and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Myocardial infarction was achieved by permanent ligation of the proximal left anterior descending artery (LAD). The ligation or sham surgery was performed on 12-week-old male mice by a technician who was blinded to the genotype[218]. Infarct size was visualized using Triphenyl Tetrazolium Chloride (TTC) (Sigma) staining and Masson's trichrome staining. The procedures for IR surgery with 30-minute occlusion protocol were similar to the MI surgery except that a piece of polyethylene tubing was placed on the LAD to minimize vessel damage[219]. The IR protocol was validated by Evans blue perfusion and electrocardiogram (ECG). Mice were sacrificed with intraperitoneal injection with ketamine (100 mg/kg) and xylazine (10 mg/kg) cocktail, and the heart tissue and bone marrow were collected.

Human tissue from non-failing control hearts and failing post-MI hearts were collected from Human Organ Procurement and Exchange program (HOPE) and Human Explanted Heart Program (HELP) respectively, with ethical approval from the Mazankowski Alberta Heart Institute and the Institutional Ethics Committee[218]. Informed and signed consents were obtained from all participants; and our study conformed to the principles outlined in the Declaration of Helsinki.

4.2.2. Echocardiography

Noninvasive transthoracic echocardiography was performed on mice anesthetized with 1.5% isoflurane in O_2 using Vevo 3100 (Visualsonics). Conventional measurements and speckle-tracking strain analysis were carried out[218,221]. Global peak systolic strain was calculated as the average of 6 standard anatomical segments.

4.2.3. Immunofluorescence

Immunofluorescence staining was performed using established protocols[218]. Wheat Germ Agglutinin (WGA, Invitrogen) staining was performed to outline cardiomyocytes. Fluorescein-conjugated Lectin (Vectorlabs) intravital perfusion was performed to identify the functional vasculature[218]. Fragmented DNA of apoptotic cells was detected using the DeadEnd Fluorometric Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-End Labeling (TUNEL) System (Promega) according to instructions.

4.2.4. Endothelial Cell Culture and Bead Angiogenesis Assay

Human umbilical vein endothelial cells (HUVECs) and human coronary artery endothelial cells (HCAECs, ATCC) were used between passage 3 to 7. Endothelial cells cultured to 70-80% confluence were transfected with small interfering RNA against human PI3Kβ (sip110β) or scrambled small interfering RNA (s-siRNA) for 48 hours to test the effects of genetic ablation of PI3Kβ on endothelial cells. Pharmacological inhibition of PI3Kβ using the PI3Kβ-specific inhibitor TGX-221 (500 nmol/L, Cayman Chemical) was used on endothelial cells for 48 hours. Cells were starved in basal medium for 5 hours prior to stimulation with 50 ng/ml or 100 ng/ml recombinant human $VEGF_{165}$ (PeproTech) for 10 minutes. Where indicated, cells were pre-incubated with the PI3Kα-specific inhibitor BYL719 (500 nmol/L, Cayman Chemical) or the Akt inhibitor MK-2206 (1 µmol/L, APExBIO) for 1 hour before VEGF stimulation.

In vitro angiogenesis bead assay of HUVECs/HCAECs was performed as described[218]. The number of sprouts was counted using ImageJ software, and at least 20 beads per independent experiment were analyzed.

4.2.5. Adult Cardiomyocyte Isolation, Culture, and Stretching

Adult murine left ventricular cardiomyocytes were isolated from isoflurane (2%)-anesthetized mice; and the isolated cardiomyocytes were cultured and stretched as described[224].

Plated cardiomyocytes were cyclically stretched at 1Hz with an elongation of 5% for 3 hours by Flexcell FX-5000 Tension System (Flexcell International Corp) in serum-free culture medium under a 2% CO_2 and 5% O_2 atmosphere.

4.2.6. Immunoblotting and Nuclear Fractionation

Immunoblotting and nuclear fractionation was performed as previously described[218,226]. Histone H3 and GAPDH (Cell Signaling) were used as nuclear and cytosolic markers, respectively.

4.2.7. RNA Sequencing and TaqMan RT-PCR

RNA isolation and RNA sequencing were performed as described[274]. Total RNAs from left ventricles (3 left ventricles/group) were extracted. Data were analyzed using WebGestalt, Protein ANalysis THrough Evolutionary Relationships (PANTHER) classification system (Pantherdb.org), and Ingenuity Pathway Analysis. RNA expression levels were examined by TaqMan real-time polymerase chain reaction (RT-PCR) as described[218]. The expression levels of myocardial disease markers, including atrial natriuretic peptide (*Anp*), brain natriuretic peptide (*Bnp*), and β-myosin heavy chain (*β-Mhc*), were examined.

4.2.8. Statistical Analysis

Statistical analyses were carried out using SPSS Statistics 24 software, and statistical significance was defined as $p < 0.05$ (two-sided). Continuous data were presented in scatter plots with mean ± SEM. The differences between the two groups were evaluated using independent t-test or Mann-Whitney U test after normality examination. One-way ANOVA or Kruskal-Wallis test with pairwise comparisons were used in studies with more than two groups based on the normality of the data. Survival data were presented as the Kaplan-Meier plots and the log-rank test was used to evaluate the statistical significance.

4.3. Results

4.3.1. Akt phosphorylation and PI3Kβ are elevated in ischemic hearts

Because of the vital role of cardiac Akt in injury repair[275], we examined the protein levels of Akt in post-MI murine heart and found increased Akt phosphorylation, especially at threonine-308, in the infarct and peri-infarct area (Figure 4.1A). As one of the upstream regulators of Akt activation, the p110β protein level was assessed next to evaluate the effect of MI on PI3Kβ. We observed an increase in p110β level in the infarct and peri-infarct area, and to a lesser extent, in the non-infarct area (Figure 4.1A). Importantly, explanted human hearts showed largely similar trends of phospho-Akt and p110β levels following MI (Figure 4.1B), suggesting a conserved mechanism of upregulation of PI3Kβ following myocardial ischemia. Immunofluorescence analysis showed that in both murine and human hearts, PI3Kβ is expressed in both endothelial cells and cardiomyocytes, and while PI3Kβ was constitutively expressed in endothelial cells, it was highly upregulated and localized to the nuclei in cardiomyocytes (Figure 4.1C and 4.1D). These findings suggest that PI3Kβ could have specific and distinct functions in endothelial cells and cardiomyocytes. Thus, we examined the function of PI3Kβ in endothelial cells and cardiomyocytes separately in response to MI and myocardial IR injury using genetically modified mice.
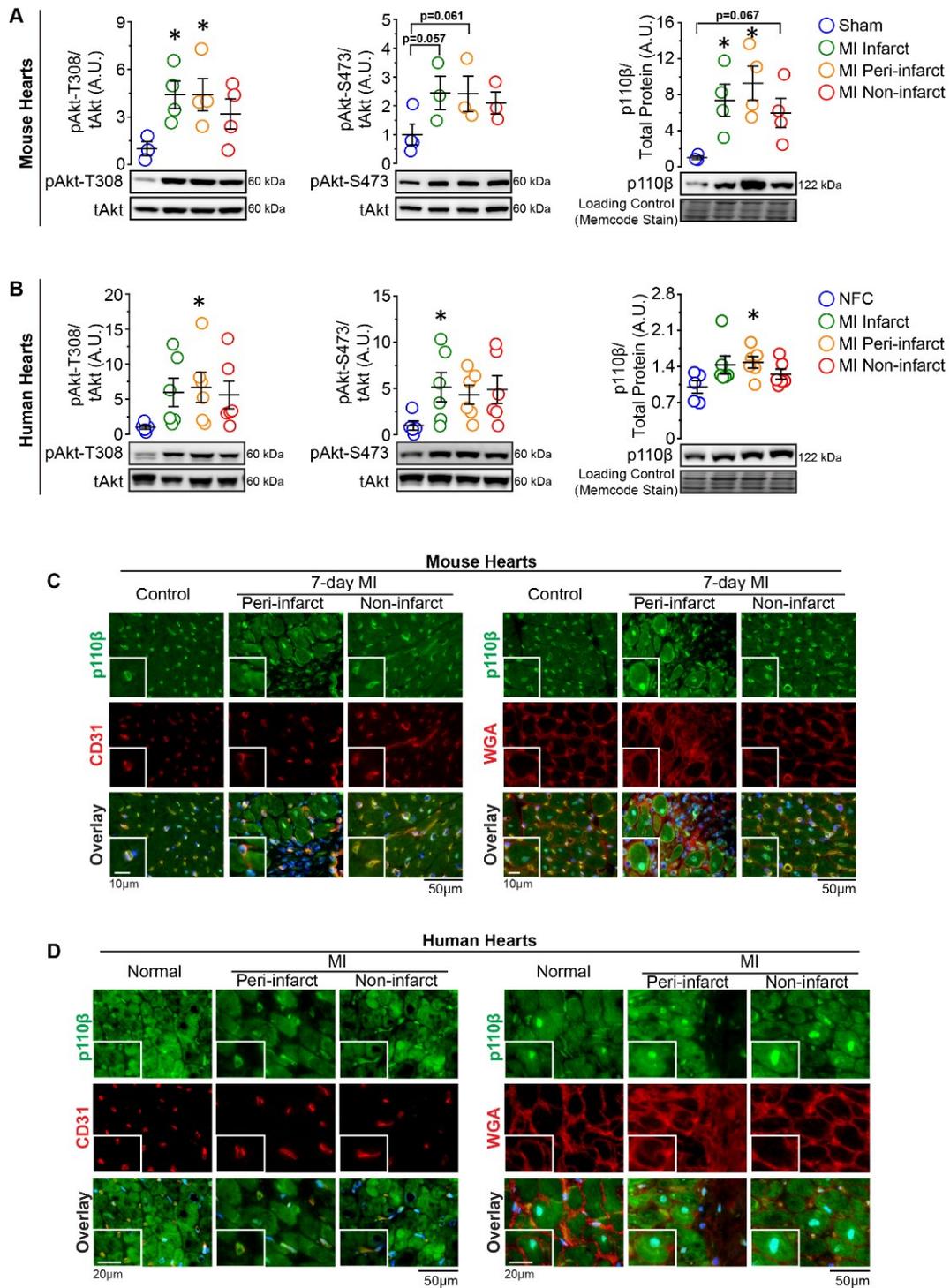
Figure 4. 1. Catalytic isoform of PI3Kβ-p110β is increased in post-MI murine and human hearts and expressed both in endothelial cells and cardiomyocytes. (A,B) Western blot analysis of p110β and Akt levels on 7-day post-sham/MI mouse hearts and on non-failing and post-MI failing human hearts. **(C,D)** Immunofluorescence images of PI3Kβ in the heart with endothelial marker-CD31, WGA outlining cardiomyocytes, and DAPI marking nuclei on mouse and human hearts. *P<0.05 vs sham/NFC group.

4.3.2. Endothelial PI3Kβ inactivation improves cardiac function and remodeling after MI

To gain insight into the role of endothelial PI3Kβ in post-MI remodeling, we generated p110βTie2 mice in which endothelial PI3Kβ was conditionally and partially deleted, producing truncated PI3Kβ protein lacking catalytic activity[94]. Successful genetic inactivation of p110β in p110βTie2 was confirmed by PCR analysis with truncated PI3Kβ gene expression without affecting hematopoietic cells (Figure 4.2A). Both p110βTie2 and littermate control (p110βFlx) mice showed similar body weight, heart weight, left ventricular weight, and levels of p110β, p110α, and phospho-Akt in whole-heart protein extracts (Figure 4.2B and 4.2C).

p110βTie2 and littermate controls were randomly and blindly assigned to sham operation or MI induction. While sham-operated groups had similar cardiac function, post-MI p110βTie2 exhibited higher survival rate (92% vs. 74%) by day 7, with improved post-MI cardiac function reflected by maintained left ventricular end-diastolic and end-systolic dimensions, greater ejection fraction and enhanced regional systolic function (Figure 4.2D and 4.2E). Consistent with the preservation of post-MI cardiac function, p110βTie2 hearts showed lower expression levels of myocardial disease markers with equivalent hypertrophy in both genotypes (Figure 4.2F and 4.2G). While initial post-MI infarct size and apoptotic level were comparable between genotypes, p110βTie2 showed reduced infarct expansion on day 7 and absence of post-MI pulmonary edema (Figure 4.3A-D). Moreover, even though sham-operated hearts and the non-infarct area of post-MI hearts displayed similar vascular density between genotypes, p110βTie2 showed increased vascular density in the infarct and peri-infarct areas, confirmed by endothelial marker staining and lectin *in vivo* perfusion for the detection of functional vessels (Figure 4.3E). Since PI3K/Akt/eNOS and Erk1/2 pathways are critical mediators of cardioprotection[276], we examined the levels of phospho-Akt and phospho-Erk1/2 and found both were increased in post-MI p110βTie2 hearts compared to control, while phospho-eNOS was significantly increased in the infarct and remote areas (Figure 4.3F). Taken together, these findings demonstrate that catalytic inactivation of endothelial PI3Kβ resulted in marked cardioprotection following MI associated with preserved vascular density and increased Akt activation in the ischemic area.
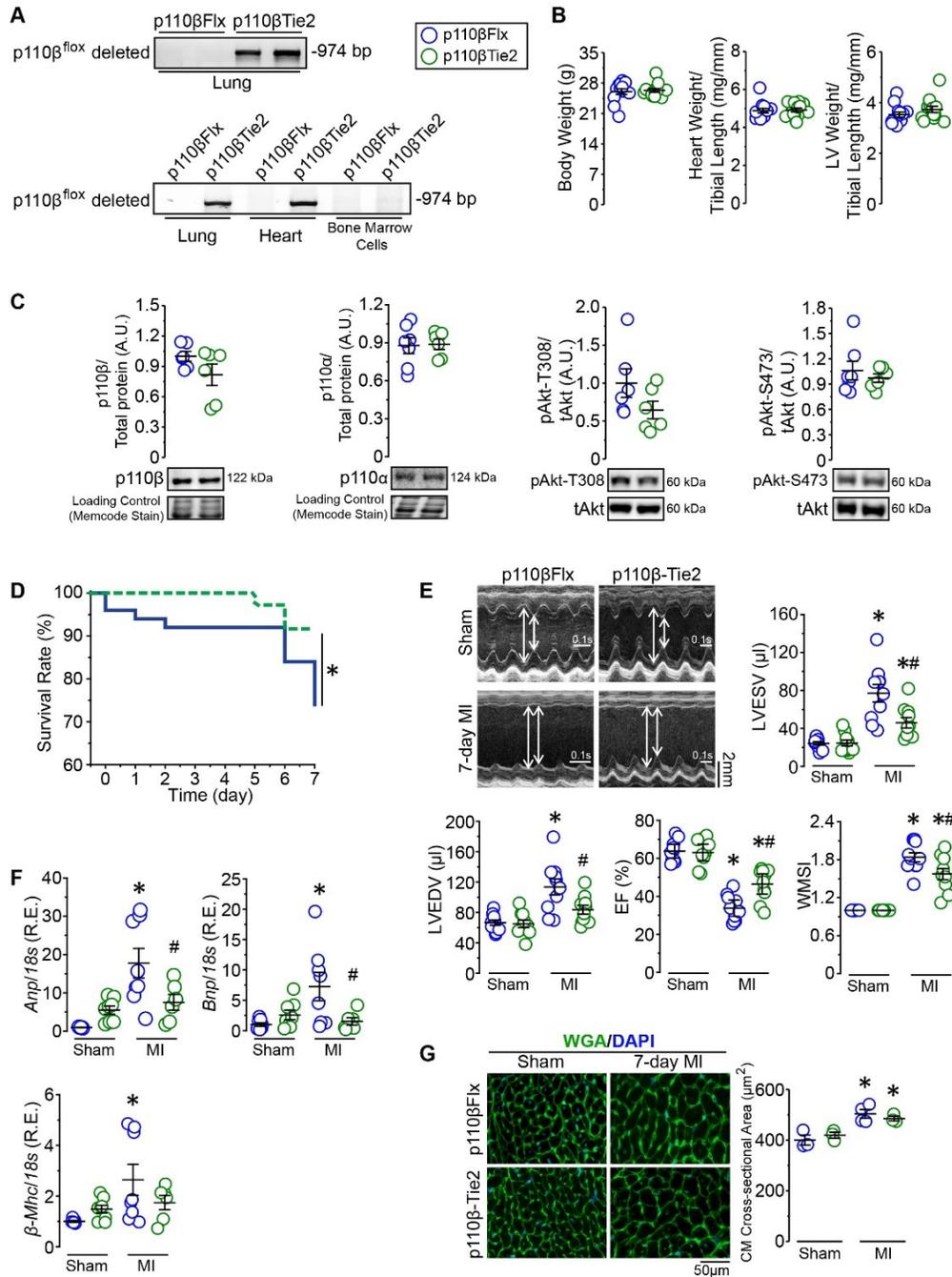
Figure 4. 2. Inactivation of endothelial PI3Kβ preserves cardiac function after MI with higher survival rate. (A) PCR analysis amplifying a fragment of the p110β allele after Cre-mediated excision of exons 21 and 22. **(B)** Body weight, heart weight, and left ventricular weight from adult mice. **(C)** Western blot analysis and representative images of p110β, p110α, and Akt protein levels. **(D)** Kaplan-Meier survival curve of mice following MI surgery. **(E)** Echocardiographic images showing ventricular morphology and analysis of left ventricular end-systolic and end-diastolic volume (LVESV and LVEDV), ejection fraction (EF), and wall motion score index (WMSI). **(F)** mRNA relative expression (R.E.) level of atrial natriuretic peptide (*Anp*), brain natriuretic peptide (*Bnp*), and β-myosin heavy chain (*β-Mhc*) with *18s* as controls. **(G)** Wheat Germ Agglutinin (WGA) analysis on 7-day post-operated hearts with DAPI staining. *P<0.05 vs sham group or vs indicated group; #P<0.05 vs p110βFlx.
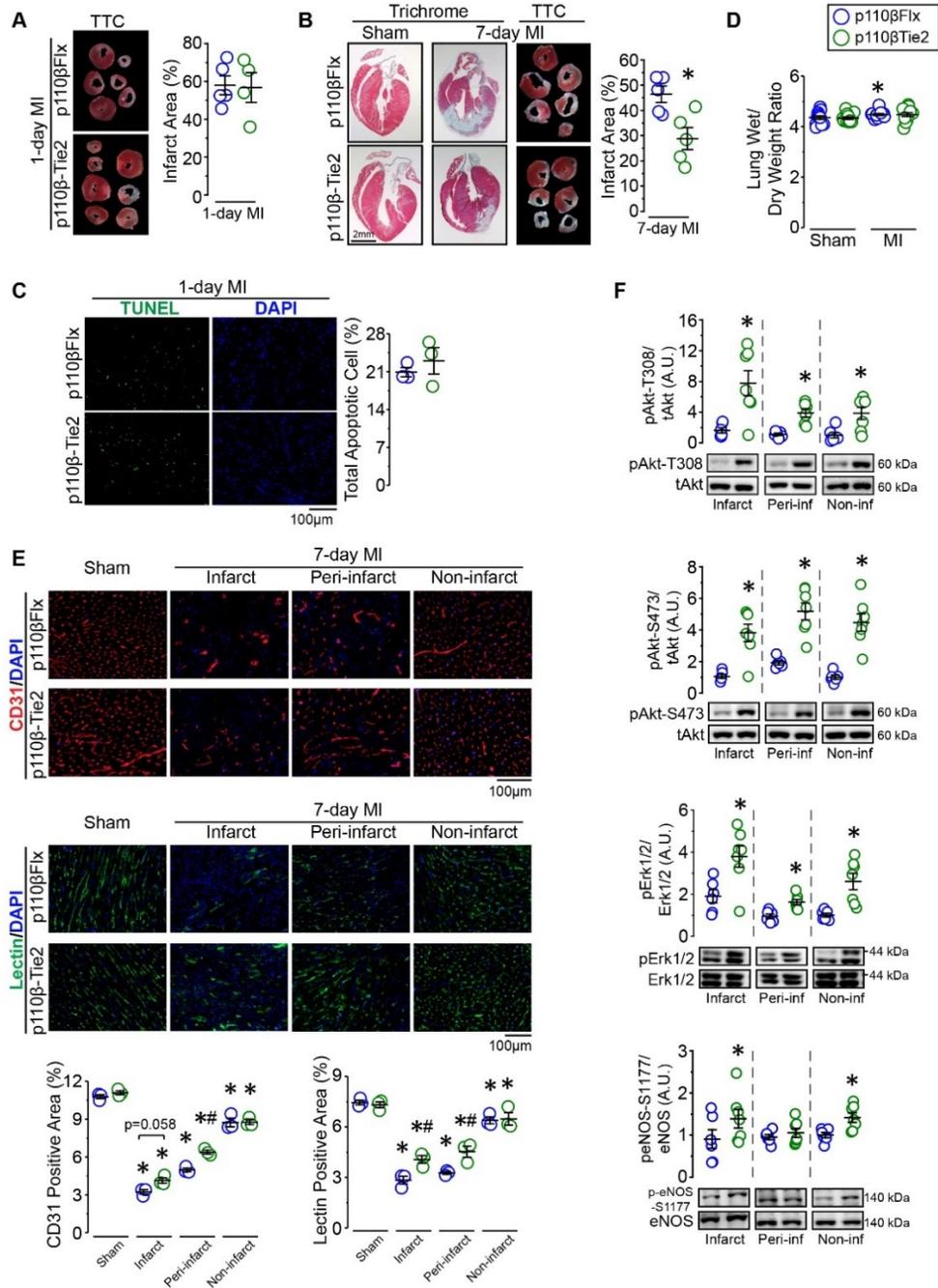
Figure 4. 3. Inactivation of endothelial PI3Kβ protects the heart against MI by preserving vasculatures and increasing Akt activity in the ischemic areas. (A,B) Infarct size analysis from triphenyl tetrazolium chloride (TTC)-stained sections and representative images of trichrome histological staining of hearts. **(C)** Representative immunofluorescence images of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and DAPI on 1-day post- MI hearts. **(D)** Pulmonary edema evaluated using lung wet/dry weight ratio at day 7 after sham and MI operation. **(E)** Immunofluorescence analysis of endothelial marker-CD31 and endothelial marker-lectin via in vivo perfusion method. **(F)** Western blot analysis of Akt, Erk1/2, and NOS protein levels in different areas from 7-day post-MI hearts. *$P < 0.05$ vs sham group or vs indicated group; #$P < 0.05$ vs p110βFlx.

4.3.3. Loss of PI3Kβ activity promotes VEGF-induced PI3Kα/Akt signaling and angiogenic sprouting in endothelial cells

The VEGF/VEGF receptor axis plays a crucial and well-established role in vascular survival and angiogenesis through PI3Kα/Akt signaling[276,277]. This was also supported by our findings in human umbilical vein endothelial cells (HUVECs) that Akt activation was required for VEGF-induced angiogenic sprouting using Akt inhibitor (MK-2206), VEGF-stimulated Akt activation largely depended on the activity of PI3Kα as the PI3Kα specific inhibitor (BYL719) completely abolished the effect of VEGF-induced Akt phosphorylation without affecting p110β and p110α protein levels, and VEGF-induced eNOS activation partially relied on Akt activity (Figure 4.4A-C).
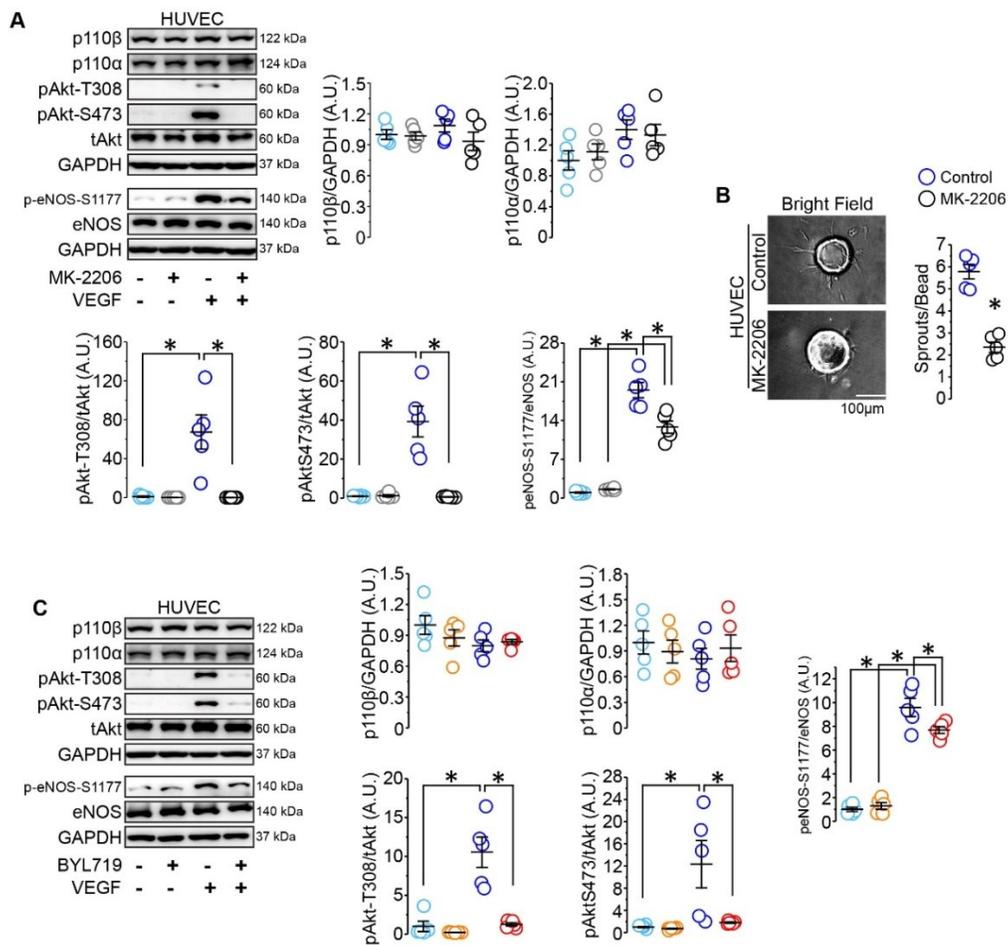
Figure 4. 4. Akt signaling is required for VEGF-induced angiogenesis which is dependent on PI3Kα. (A) Western blots showing p110β, p110α, Akt, GAPDH, and eNOS protein levels on Akt inhibitor-MK-2206-treated HUVECs. **(B)** Representative beads and quantification of sprout number in control and MK-2206-treated HCAECs. **(C)** Western blot analysis of p110β, p110α, GAPDH, Akt, and eNOS in BYL719-treated HUVEC lysates. *P<0.05 vs indicated group.

Because of the vital role of PI3Kα in VEGF-induced and Akt-mediated angiogenesis, we hypothesized that selective inactivation of PI3Kβ in endothelial cells might upregulate PI3K/Akt signaling via the PI3Kα isoform. We first tested the effects of VEGF on HUVECs with PI3Kβ-specific siRNA which showed that abrogation of PI3Kβ increased Akt and eNOS phosphorylation upon VEGF stimulation which was completely blocked by BYL-719 (Figure 4.5A-B). These results suggest that the abrogation of PI3Kβ leads to an enhanced activation of PI3Kα/Akt signaling. Next, we studied the effect of pharmacological PI3Kβ inhibition in endothelial cells on VEGF-induced Akt/eNOS activation as cardioprotective effects were observed in mice with endothelial-PI3Kβ inactivation (p110βTie2). Consistent with our animal and PI3Kβ-deleted endothelial cell data, the PI3Kβ specific inhibitor (TGX-221) potentiated VEGF-induced Akt activation in HUVECs, which was completely dependent on intact PI3Kα signaling (Figure 4.5C-D).

To address whether these effects exist in cardiac endothelial cells, human coronary artery endothelial cells (HCAECs) were examined the effect of PI3Kβ inhibition on VEGF/PI3K/Akt signaling. Immunofluorescence analysis confirmed the expression of PI3Kβ in cardiac endothelial cells (Figure 4.6A). Consistent with our findings in HUVECs, PI3Kβ inhibition in HCAECs resulted in a striking increase in Akt and eNOS phosphorylation upon VEGF stimulation without affecting p110α and p110β protein levels, which were suppressed by PI3Kα inhibition (Figure 4.6B-C). Since the PI3K/Akt pathway is critical in angiogenesis and increased vasculature was detected in the ischemic area in post-MI p110βTie2 hearts, we performed the angiogenic bead assay to test the effect of PI3Kβ inhibition on angiogenic sprouting in HCAECs. Coinciding with increased Akt activation, PI3Kβ inhibition potentiated angiogenic sprouting (Figure 4.6D). Taken together, these data suggest that inhibiting PI3Kβ activity enhances VEGF-mediated Akt activation via the PI3Kα isoform resulting in increased angiogenic response in cardiac endothelial cells.
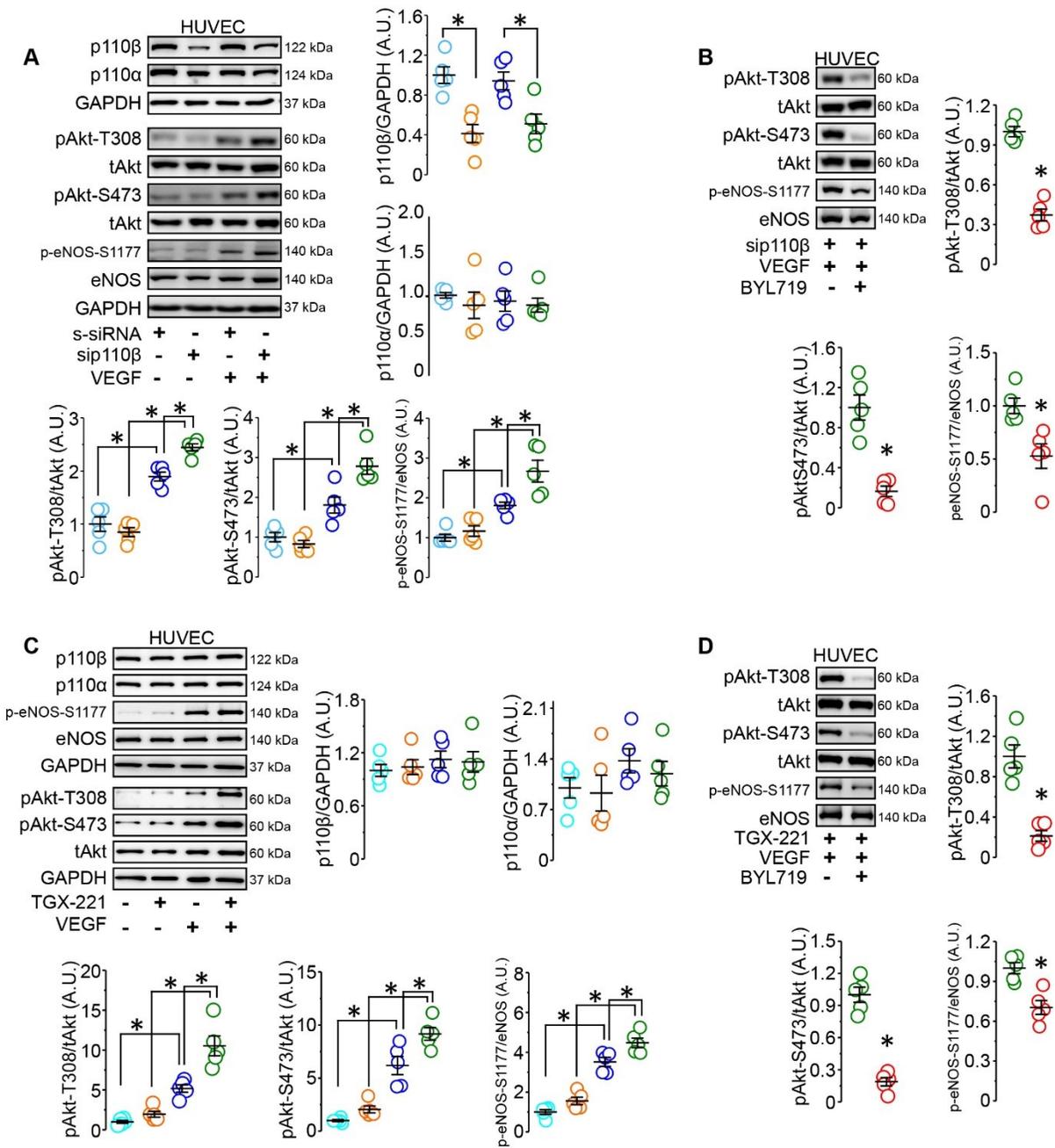
Figure 4. 5. Loss or inhibition of PI3Kβ in HUVECs elevates VEGF-stimulated PI3Kα/Akt/eNOS signaling. (A) Western blot analysis of p110β, p110α, Akt, and eNOS in PI3Kβ-deficient HUVECs. **(B)** Western blot analysis of Akt and eNOS in PI3Kβ-deficient HUVECs with and without PI3Kα-specific inhibitor, BYL719. **(C)** Western blots demonstrating the effect of VEGF on p110β, p110α, GAPDH, Akt, and eNOS protein levels in TGX-221-treated HUVECs. **(D)** Western blot analysis of Akt and eNOS in TGX-221-treated HUVECs with and without BYL719. *P<0.05 vs indicated group.
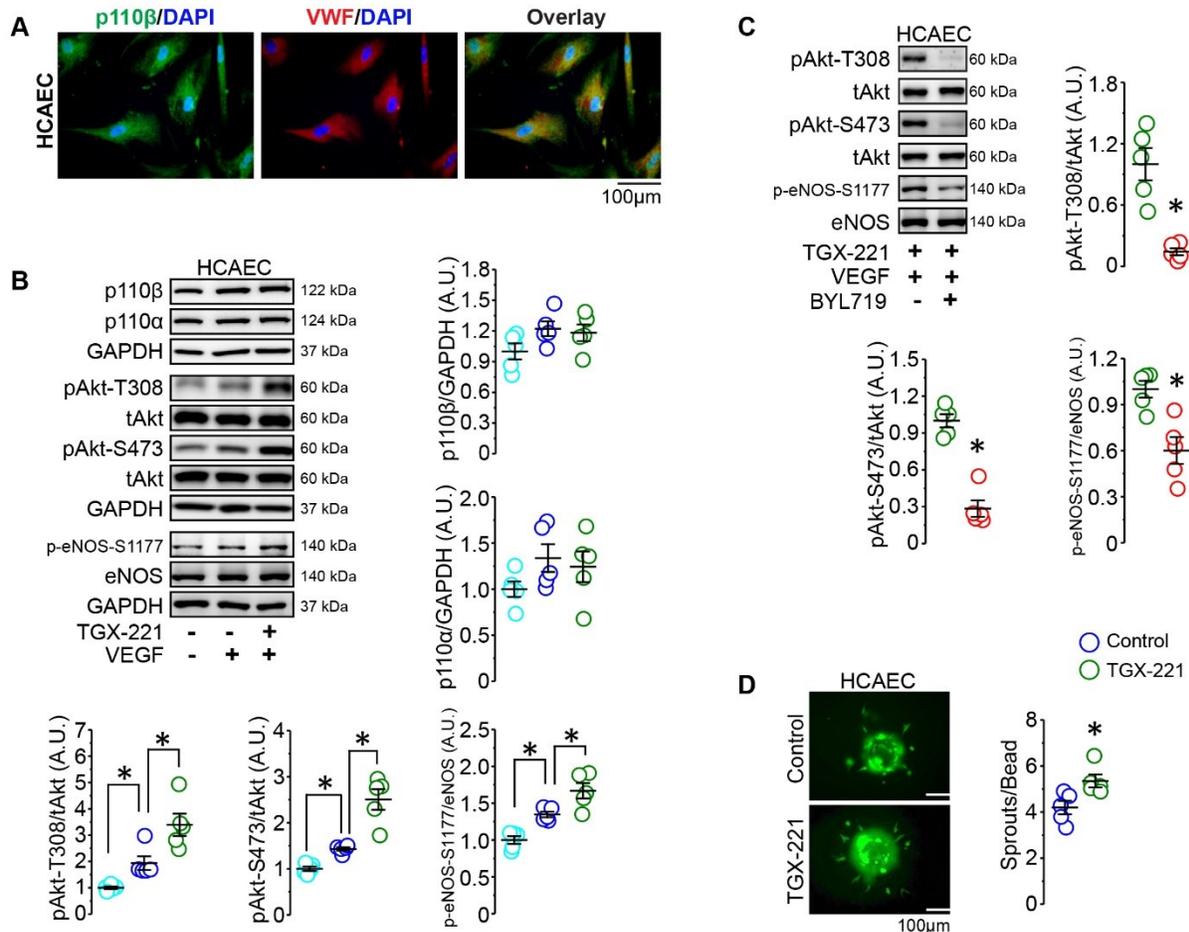
Figure 4. 6. Inhibition of PI3Kβ in human cardiac endothelial cells enhances PI3Kα/Akt/eNOS signaling and angiogenesis. (A) HCAEC stained with p110β, Von Willebrand factor (VWF), and DAPI. **(B)** Western blot analysis of p110β, p110α, GAPDH, Akt, and eNOS in HCAECs. **(C)** Western blot analysis of Akt and eNOS in TGX-221-treated HCAECs with and without BYL719. **(D)** Representative beads and quantification of sprout number in control and TGX-221-treated HCAECs. *P<0.05 vs indicated group.

4.3.4. Inactivation of PI3Kβ in cardiomyocytes exacerbates cardiac dysfunction following MI

We next evaluated the role of cardiomyocyte-PI3Kβ in post-MI remodeling using p110β-αMHC mice which express kinase-dead PI3Kβ specifically in cardiomyocytes. Analysis of truncated p110β gene expression confirmed the success of PI3Kβ inactivation in p110β-αMHC hearts, and the adult p110β-αMHC mice were viable and fertile with comparable body weight to littermate controls-p110βFlx (Figure 4.7A-B). The protein level of p110β was reduced in p110β-αMHC hearts without altering whole-heart baseline phospho-Akt levels (Figure 4.7C). However, at day 7 post-MI, p110β-αMHC mice exhibited a trend of increased mortality compared to controls (62% vs. 76%), with increased left ventricular dimensions, deteriorated systolic

function, and worsened regional systolic function (Figure 4.7D-E). Chamber dimensions and cardiac function did not differ between genotypes in sham-operated groups (Figure 4.7E).
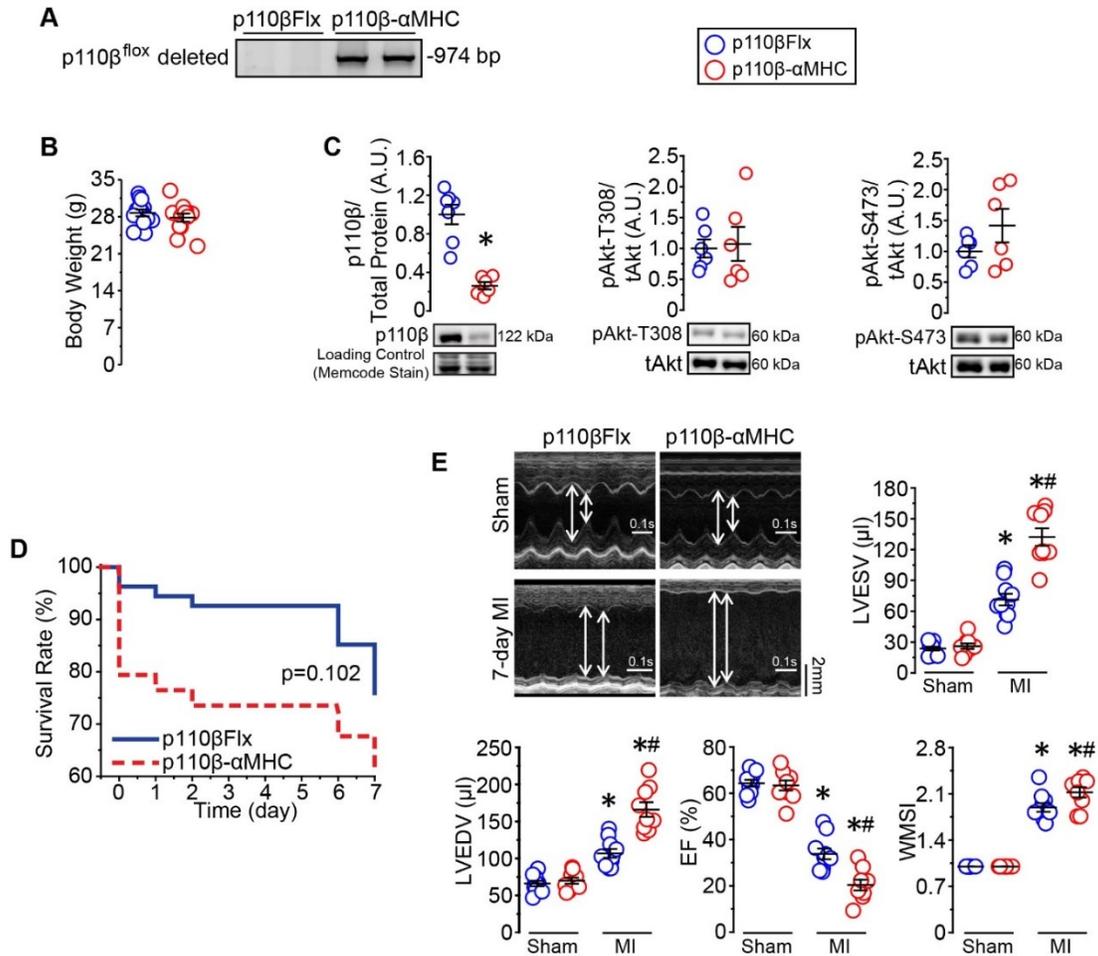
Figure 4. 7. Cardiomyocyte-specific PI3Kβ inactivation exacerbates cardiac dysfunction after MI. **(A)** PCR analysis amplifying a fragment of the p110β allele with the deletion of exons 21 and 22. **(B)** Body weight of p110β-αMHC and p110βFlx mice before surgery. **(C)** Western blot analysis of p110β and Akt levels in p110β-αMHC and p110βFlx left ventricular lysates. **(D)** Kaplan-Meier survival analysis in post-MI p110β-αMHC and control mice. **(E)** Echocardiographic images showing left ventricular morphology and functional analysis of left ventricular end-systolic and end-diastolic volume (LVESV and LVEDV), ejection fraction (EF), and wall motion score index (WMSI). *$P<0.05$ vs sham group or vs indicated group; #$P<0.05$ vs p110βFlx.

Consistent with worsened cardiac function, p110β-αMHC hearts showed larger infarcted area on days 1 and 7 after MI (Figure 4.8A). This functional and structural deterioration in post-MI p110β-αMHC hearts was accompanied by increased pathological hypertrophy, reduced coronary microvasculature, and strikingly increased MI-related myocardial inflammation (Figure 4.8B-D). Importantly, analysis of the canonical Akt signaling pathway displayed comparable

pAkt-T308 levels, while phosphorylation of Akt at serine-473 was slightly increased in the peri-infarct area of p110β-αMHC hearts (Figure 4.8E). These results demonstrate that in striking contrast to PI3Kβ function in endothelial cells, loss of PI3Kβ activity in cardiomyocytes resulted in increased susceptibility to ischemic injury and adverse post-MI remodeling illustrating a novel cell-dependent role of PI3Kβ signaling.
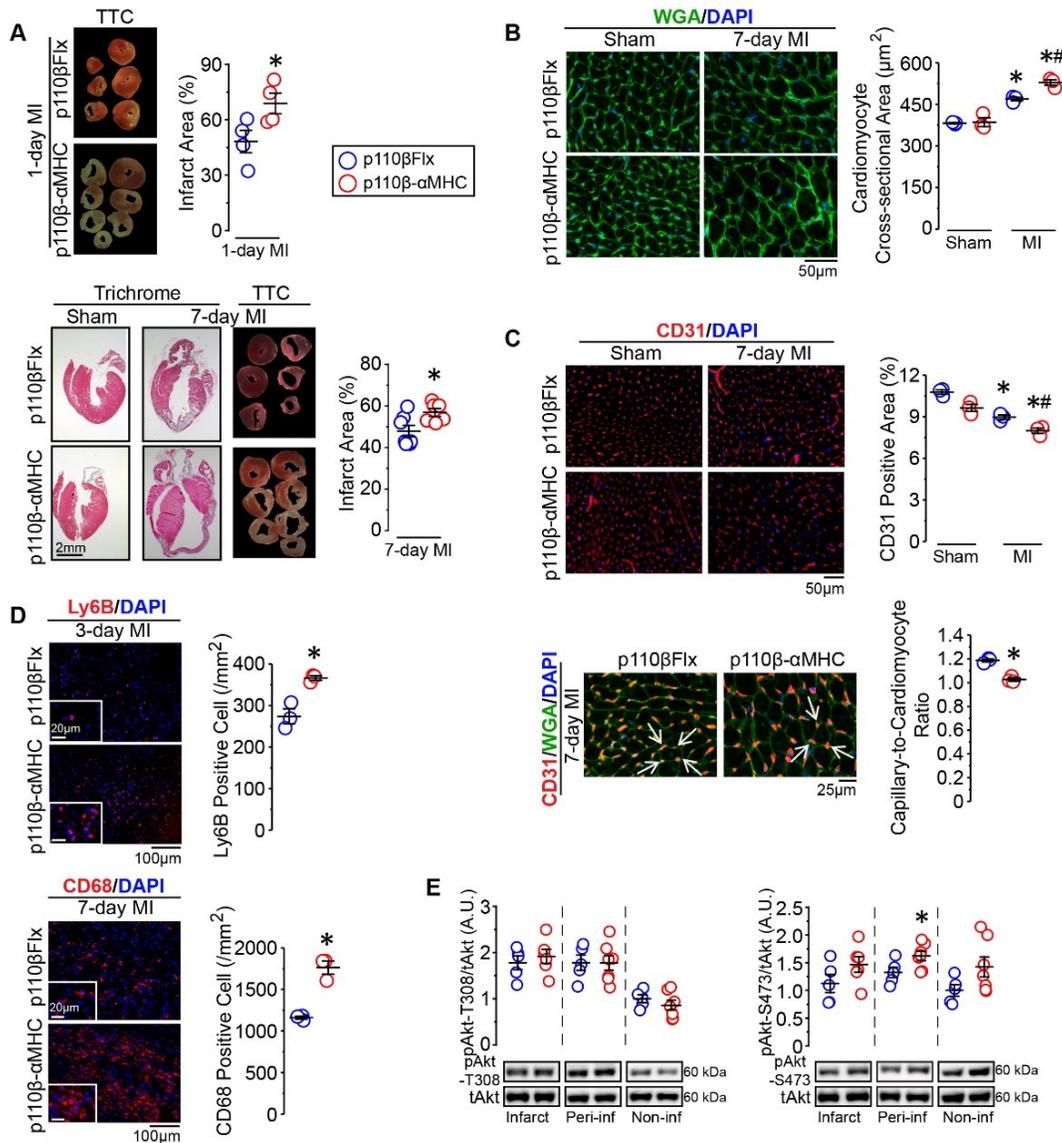
Figure 4. 8. Cardiomyocyte-PI3Kβ inactivation leads to adverse ventricular remodeling after MI. (A) Trichrome histological-stained images and triphenyl tetrazolium chloride (TTC)-stained images and infarct size quantification on post-MI hearts. **(B)** Wheat Germ Agglutinin (WGA) immunofluorescence staining outlining cardiomyocyte size. **(C)** Vascular density and capillary-to-cardiomyocyte ratio testing by CD31 and WGA immunofluorescence staining on post-surgery hearts. **(D)** Neutrophils identified by Ly6B and macrophages marked by CD68 immunofluorescence staining. **(E)** Western blot analysis of Akt protein level. *P<0.05 vs sham group or vs indicated group; #P<0.05 vs p110βFlx.

4.3.5. Cardiomyocytes with PI3Kβ deficiency are prone to hypoxia-induced cell death: role of PI3Kβ in the regulation of myocardial gene expression

Given the increase in infarct size in p110β-αMHC hearts, we hypothesized that PI3Kβ prevents cardiomyocyte death. Immunofluorescence and Western blot analysis demonstrated increased apoptosis in post-MI p110β-αMHC hearts, characterized by elevated apoptotic cell number and cleaved caspase 3 protein levels (Figure 4.9A). Since cardiomyocytes are not the only cell types to undergo apoptosis under MI, we combined TUNEL and WGA staining to evaluate apoptotic cardiomyocytes which confirmed an increase in apoptotic cardiomyocytes in the infarct and peri-infarct area (Figure 4.9B). To elucidate the molecular mechanisms underlying the increased susceptibility of p110β-αMHC cardiomyocytes to apoptosis, we analyzed cell death proteins at baseline in left ventricular samples. Pro-apoptotic proteins, including full-length caspase 3, full-length caspase 8, Bax, and Bak, were upregulated in p110β-αMHC; meanwhile RIP3, a critical determinant of necrosis, but not RIP1, was also upregulated (Figure 4.9C). To determine the effect of the loss of PI3Kβ in cardiomyocytes, we isolated adult cardiomyocytes and examined the role of PI3Kβ in hypoxia-induced cell death under cyclic mechanical stretch. The results showed that cell viability was decreased in p110β-αMHC cardiomyocytes compared with control cardiomyocytes in response to 3-hour cyclical stretch under hypoxic condition (5% O_2) (Figure 4.9D). In line with these data, we observed higher creatine kinase level in the culture media, a marker of cardiomyocyte death, and an increase in the number of apoptotic cardiomyocytes in p110β-αMHC cardiomyocytes (Figure 4.9E-F). Thus, cardiomyocytes with compromised PI3Kβ activity develop an intrinsic susceptibility to cell death in response to acute ischemic and hypoxic stress.

Western blot analysis demonstrated that PI3Kβ was present in the nuclear fraction of left ventricles, and immunostaining on isolated adult cardiomyocytes confirmed the presence of PI3Kβ in the nuclei, and both cytosolic and nuclear fractions were decreased in p110β-αMHC hearts (Figure 4.10A). Surprisingly, in p110β-αMHC hearts, phospho-Akt level was downregulated in the nuclear fraction, but not in the cytosolic fraction, without alteration in the protein levels of FoxO1 and FoxO3a, which are known down-stream effectors of Akt phosphorylation (Figure 4.10B). Taken together, these findings indicate that deficiency of cardiomyocyte-PI3Kβ results in decreased nuclear Akt phosphorylation which might affect nuclear activity independent of FoxO transcription factors, leading to a pro-cell death phenotype in cardiomyocytes.
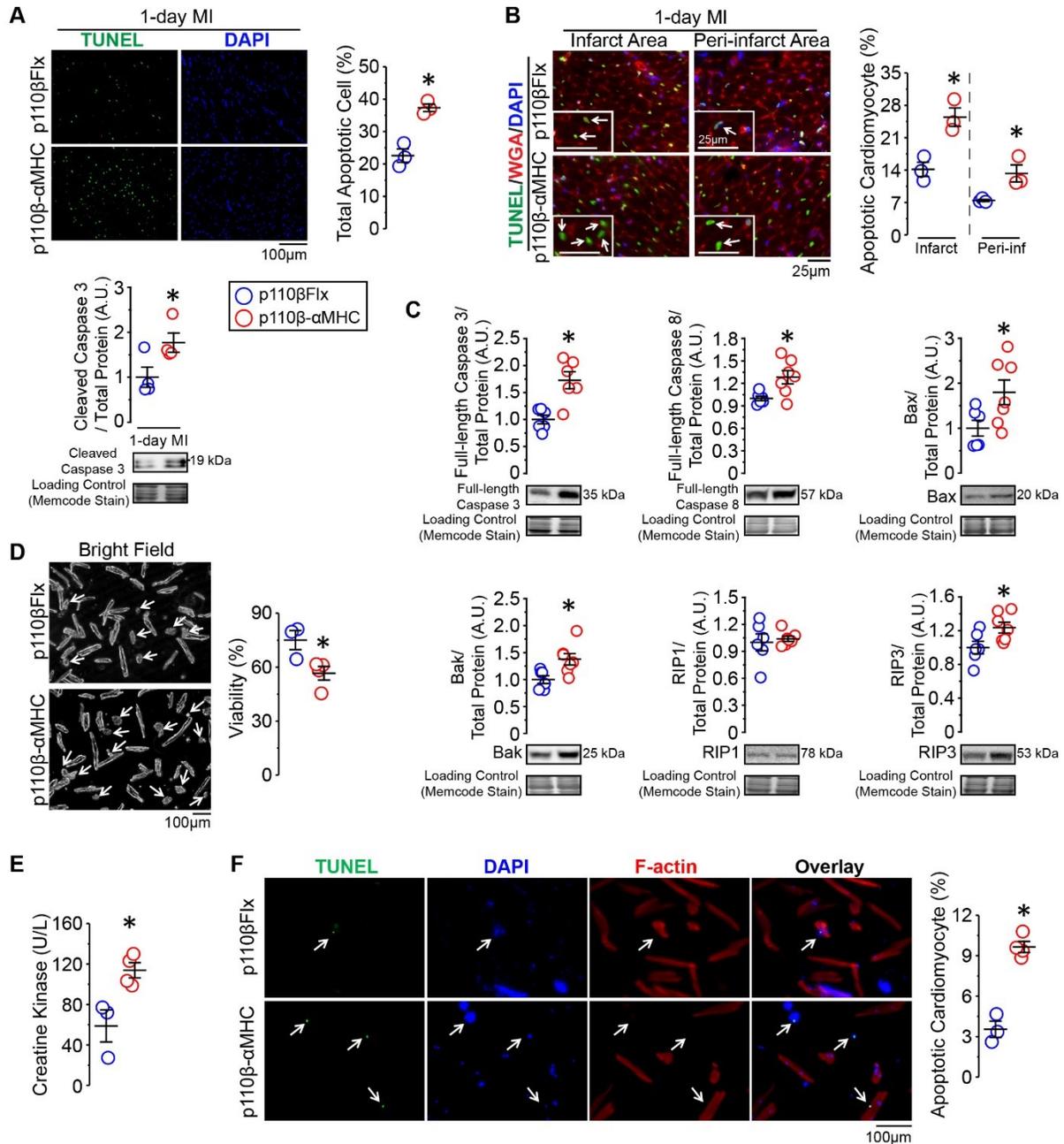
Figure 4. 9. Inactivation of cardiomyocyte-PI3Kβ sensitizes cardiomyocytes to cell death by increasing pro-cell death proteins expression, leading to increased post-MI cell death. (A) TUNEL and DAPI immunofluorescence analysis for apoptotic cells and western blot analysis for cleaved caspase 3 on 1-day post-MI hearts. **(B)** Combined WGA, TUNEL, and DAPI immunofluorescence staining to highlight apoptotic cardiomyocytes. **(C)** Western blot analysis for baseline protein levels of full-length caspase 3, full-length caspase 8, Bax, Bak, RIP1, and RIP3 in left ventricle lysates from p110β-αMHC and control mice. **(D)** Representative right field images and cell viability evaluation after stretching. **(E)** Evaluation of creatine kinase level in media from cultured cardiomyocytes. **(F)** TUNEL staining from apoptotic cardiomyocytes with F-actin staining. *P<0.05 vs indicated group.
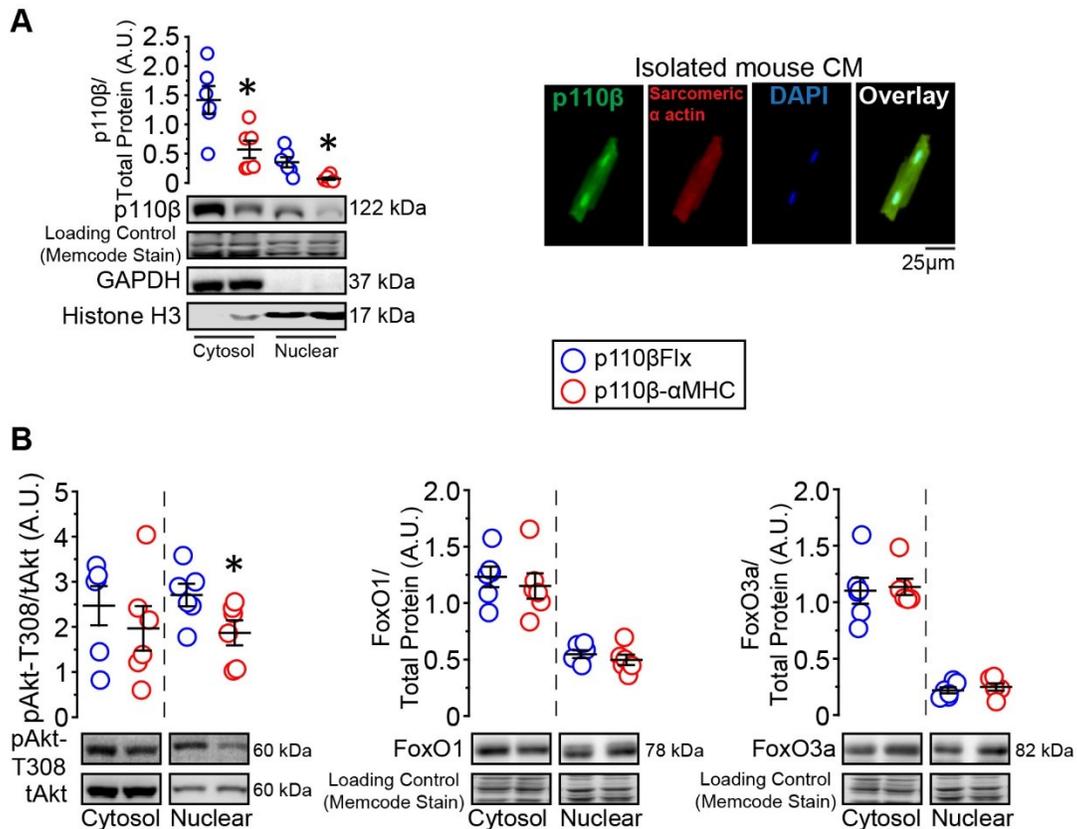
Figure 4. 10. PI3Kβ is expressed in cardiomyocyte nuclei and loss of PI3Kβ is associated with decreased nuclear Akt phosphorylation. (A) Nuclear fractionation analysis for p110β, GAPDH and Histone H3 in p110β-αMHC and p110βFlx left ventricular lysates and immunofluorescence images of p110β and sarcomeric α actin on isolated murine cardiomyocytes. **(B)** Fractionation analysis for Akt, FoxO1, and FoxO3a in p110β-αMHC and p110βFlx left ventricular lysates. *P<0.05 vs p110βFlx in A and vs indicated group in B.

To better define the involvement of PI3Kβ in transcriptional control, we performed RNA sequencing to clarify the differences in global gene expression profiles in control and p110β-αMHC left ventricles, which revealed 1057 up-regulated genes and 838 down-regulated genes in p110β-αMHC hearts, affecting the pathways associated with metabolism, cell cycle, and chemokine signaling (Figure 4.11A-B). Notably, among the affected genes in the p110β-αMHC hearts, genes associated with cell death were prominently upregulated, while genes related to the regulation of metabolic processes were downregulated (Figure 4.11C). Among the upregulated genes related to biological processes, cellular processes were altered, while protein tyrosine kinases signaling pathway showed minimal changes (Figure 4.11D). Moreover, apoptosis and inflammatory signaling pathways were dramatically up-regulated in p110β-αMHC (Figure 4.11E) which is consistent with our observations demonstrating excessive apoptosis and inflammation in the post-MI p110β-αMHC hearts. Ingenuity Pathway Analysis identified

activation of *Creb1*, *Smad3*, *Mkl1*, and *Nr3c2* transcription factors; furthermore, *Smad7*, *Tfam*, *Ppara*, and *Klf15* transcription factors were inhibited in p110β-αMHC hearts (Figure 4.11F). Several of these transcriptional regulators are known to be associated with detrimental post-infarct outcomes (*Mkl1, Nr3c2, Smad,* and *Tfam*), as well as with regulation of cardiac metabolism contributing to infarct healing (*Ppara* and *Klf15*)[278-283]. Our data suggest that PI3Kβ signaling in cardiomyocyte is required for protection against ischemic injury and nuclear Akt contributes to this protection.
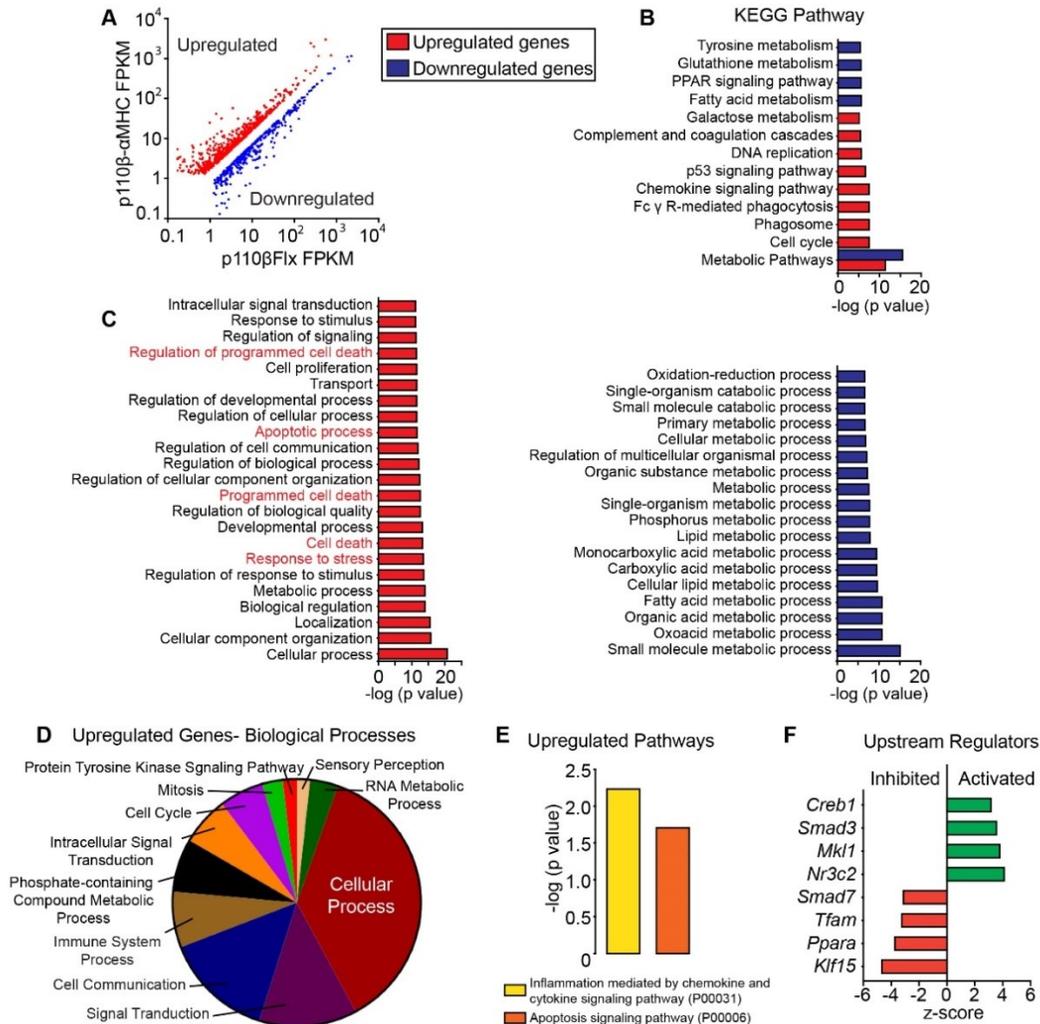
Figure 4. 11. Cardiomyocyte-PI3Kβ inactivation alters gene expression in the heart, which is associated with increased apoptotic signaling. (A) Scatterplot of significantly differentially expressed genes after RNA-seq analysis on p110β-αMHC and p110βFlx left ventricles. **(B)** KEGG pathway enrichment analysis of RNA-seq results for significantly altered genes. **(C)** WebGestalt enrichment analysis for disrupted pathways on significantly upregulated and downregulated genes in biological processes. **(D)** Panther GO analysis showing subcategories of differential gene expression in biological processes. **(E)** Significantly altered signaling pathways identified by Panther signal transduction pathways analysis. **(F)** Potentially altered upstream transcription factors identified by Ingenuity Pathway Analysis.

4.3.6. PI3Kβ has divergent effects in ECs and CMs facing ischemia-reperfusion (IR) injury

We next examined whether inactivation of p110β in endothelial cells or cardiomyocytes would affect cardiac performance after IR injury which comprises of ischemic and reperfusion injury and commonly present in MI patients following myocardial reperfusion treatments[284]. IR injury was performed using 30 minutes of LAD occlusion followed by reperfusion in p110β-Tie2, p110β-αMHC, and control mice. *In vivo* Evans Blue perfusion confirmed the occlusion of the LAD as the affected myocardium remained unstained, and successful reperfusion was confirmed when this area was perfused with the dark blue dye stain after the release of LAD obstruction (Figure 4.12A). Furthermore, bipolar surface electrocardiogram obtained from these mice showed prolonged QRS and elevated ST-segment after 30 min of LAD occlusion compared to baseline electrocardiogram (Figure 4.12B), indicating the presence of myocardial ischemia.
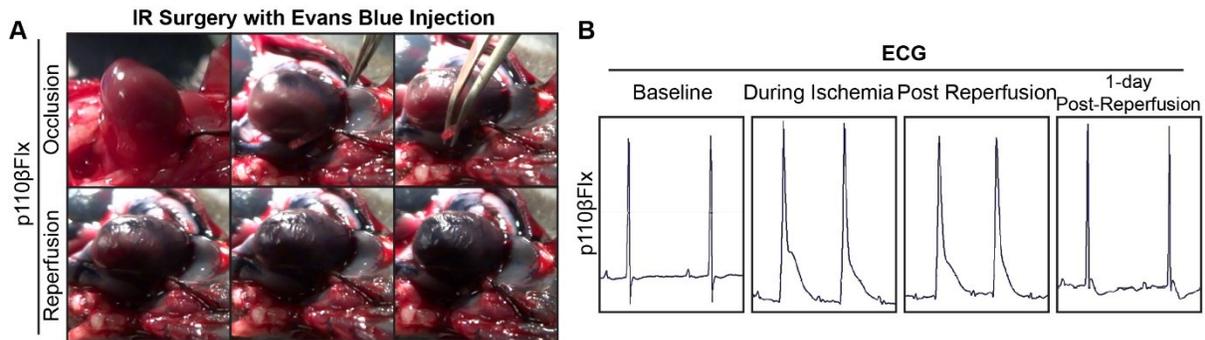
Figure 4. 12. Myocardial IR injury model is confirmed by Evans Blue perfusion and electrocardiogram. (A) Sequential images of IR surgery with Evans Blue perfusion showing the blue dye rushes into the infarcted area after the release of occlusion. **(B)** Representative electrocardiogram (ECG) of mice with IR surgery.

Myocardial strain analysis revealed better longitudinal peak systolic strain in the p110βTie2 mice at day 7 post-IR compared to control (Figure 4.13A). Consistent with improved cardiac function, p110βTie2 mice exhibited higher coronary density in the ischemic and peri-ischemic myocardium (Figure 4.13B). In contrast, at 7-day post-IR, p110β-αMHC mice displayed deteriorated cardiac function with decreased longitudinal peak systolic strain (Figure 4.13C). In addition, TUNEL staining of 3-hour post-reperfusion hearts revealed increased apoptotic cardiomyocytes in p110β-αMHC hearts (Figure 4.13D). These results clearly support the use of myocardial strain analysis to detect changes in cardiac performance and demonstrate that endothelial inactivation of PI3Kβ is cardioprotective against IR injury while PI3Kβ deficiency in cardiomyocytes enhances the susceptibility to IR injury.
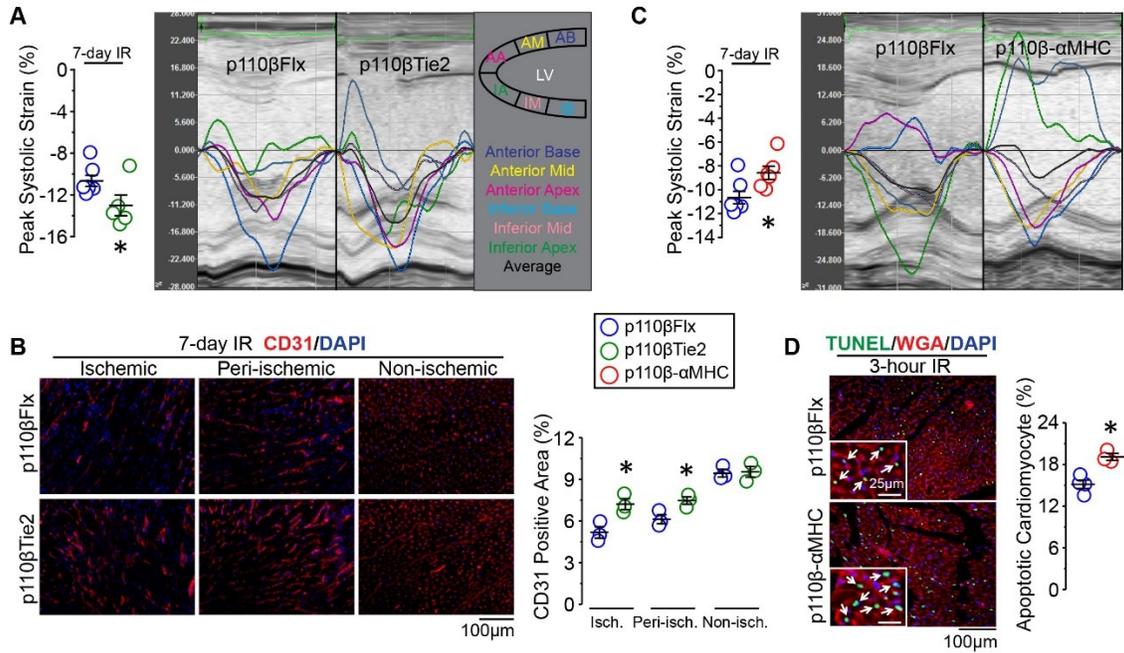
Figure 4. 13. Lack of endothelial PI3Kβ protects the heart against IR injury with increased vascular density, whilst cardiomyocyte PI3Kβ is required to maintain cardiomyocyte survival after IR. (A) Echocardiographic longitudinal strain analysis on post-IR p110βTie2 and control mice with representative longitudinal strain curve images. **(B)** Vascular density evaluated by staining endothelial marker-CD31. **(C)** Echocardiographic longitudinal strain analysis. **(D)** TUNEL staining with WGA showing apoptotic cardiomyocytes on 3-hour post-IR hearts. *P<0.05 vs indicated group in A, C, and D and vs p110βFlx in B.
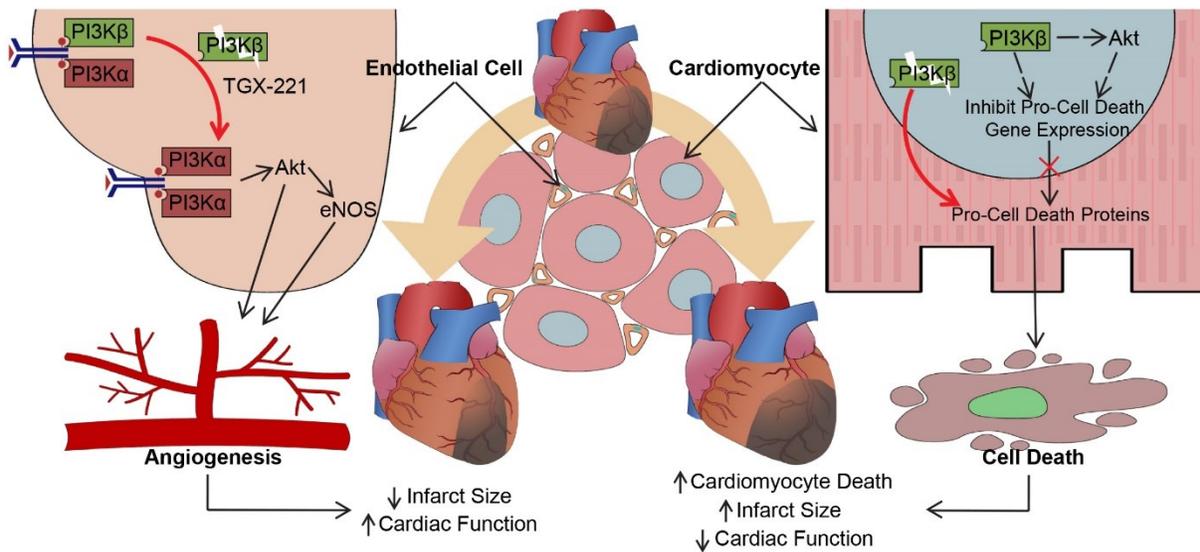
Figure 4. 14. Divergent role of PI3Kβ in endothelial cells and cardiomyocytes in the ischemic heart. Disruption of endothelial-p110β signaling enhances PI3Kα/Akt activation in the ischemic heart, promoting angiogenesis, while nuclear PI3Kβ/Akt in cardiomyocytes is required to maintain cellular homeostasis to prevent cell death facing ischemic stress.

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4.4. Discussion

Our findings reveal a novel, critical, and cell-specific role of PI3Kβ in the regulation of endothelial sprouting and cardiomyocyte survival in ischemic hearts. Using endothelial- and cardiomyocyte-specific PI3Kβ-inactivated mice which display comparable cardiac function under physiological conditions, we demonstrate that PI3Kβ plays distinct cell-specific roles in the ischemic heart. Specifically, inactivation of endothelial PI3Kβ enhances VEGF-stimulated PI3Kα/Akt/eNOS signaling and angiogenesis, reducing myocardial ischemic injury *in vivo*, whereas cardiomyocyte-specific PI3Kβ ablation disrupts cellular homeostasis with a pro-cell death profile, sensitizing cardiomyocytes to cell death following myocardial ischemia (Figure 4.14). In the heart, PI3Kβ is expressed in endothelial cells and cardiomyocytes and is upregulated following MI in both murine and human hearts confirming the PI3Kβ signaling pathway is altered in heart disease.

In striking contrast to the dispensable role of endothelial PI3Kβ in adult quiescent cardiac vasculature[189], endothelial-specific PI3Kβ inactivation leads to increased Akt phosphorylation, myocardial microvasculature preservation, cardiac function maintenance, and reduced mortality after ischemic injury. Activation of endothelial Akt/eNOS pathway is essential in VEGF-induce postnatal angiogenesis by regulating cell survival, migration, and NO release[171,263,285]. Interestingly, despite that endothelial PI3Kα drives the VEGF-induced Akt phosphorylation and following angiogenesis, the reduction or inhibition of PI3Kβ in HUVECs enhances VEGF-induced PI3Kα/Akt activation and, to a lesser extent, eNOS activation, improving angiogenic sprouting. The short-term endothelial PI3Kβ inhibition has no influence on VEGF-induced Akt activation[189,216]. However, studies on PI3Kα- or PI3Kβ-dependent cancer cells have revealed time-dependent activation of PI3K/Akt signaling in response to dominant-PI3K isoform inhibition by relieving the feedback inhibition of dominant-PI3K isoform on another isoform[286,287]. Similarly, our results suggest that inhibition of PI3Kβ in endothelial cells relieves feedback inhibition of PI3Kβ on PI3Kα amplifying VEGF-induced Akt activation and angiogenic sprouting after MI, leading to reduced infarct size, protected cardiac function, and reduced mortality.

Similarly to endothelial-PI3Kβ, cardiomyocyte-PI3Kβ is not required for postnatal cardiac development[180]. However, in response to myocardial ischemia, in contrast to the cardioprotective effects of endothelial PI3Kβ inactivation, we observe that inactivation of PI3Kβ in myocytes sensitizes them to cell death in response to ischemic injury, leading to adverse cardiac remodeling and deteriorated cardiac function. In line with our observation that considerable nuclear-PI3Kβ were present in the cardiomyocytes, cytosolic p110β/p85β complexes are known to enter the nucleus where they play a role in protecting cells against

oxidative stress-induced apoptosis and regulating DNA replication and repair[109-111]. In this framework, inactivation and/or reduction in levels of nuclear PI3Kβ should promote oxidative stress-induced apoptosis and hinder DNA repair facilitating cell death increasing size of infarct. In addition, cardiomyocyte-PI3Kβ deficiency significantly affects the transcriptional profile of the myocardium characterized by altered the expression of metabolic genes and increased the expression of programmed cell death genes, leading to the increase in pro-cell death protein levels, including caspase 3, caspase 8, Bax, Bak, and RIP3, which are associated with adverse outcomes in ischemic hearts by promoting cardiomyocyte death and increasing infarct size[15,288,289]. In line with this context, cardiac overexpression of PI3Kβ mediates cardioprotective effects in mice with MI by reducing hypoxia-induced cardiomyocyte apoptosis with increased Akt activation[211]. In this study, we found that overall Akt activation is largely unaffected by PI3Kβ deficiency either at quiescent or infarcted state, which is in line with prevailing view that PI3Kβ is not required for postnatal cardiac development[180]. However, nuclear Akt activation is diminished in PI3Kβ deficient mice, suggesting that nuclear fraction of PI3Kβ/Akt contributes to the adverse post-MI outcomes in the model. The cardioprotective function of nuclear PI3Kβ may be mediated via Akt, which is known to have multiple roles in the nucleus, such as promoting cell survival and regulating cell cycle[143]. Specifically, in the heart, nuclear accumulation of Akt inhibits cardiomyocyte apoptosis, protecting the heart against IR injury[290].

Overall, our study highlights that PI3Kβ is a versatile PI3K isoform in the heart where it has distinct roles in the cardiac endothelium versus cardiomyocytes. Inactivation of PI3Kβ in the cardiac endothelium protects the heart from ischemic injury by promoting PI3Kα/Akt signaling and angiogenesis, whereas inactivation of PI3Kβ in cardiomyocytes promotes ischemia-induced cell death by disrupting gene programs and increasing pro-cell death protein levels.

Chapter 5

Discussion

5.1. Important findings

In this dissertation, I have studied the role of PI3Kα and PI3Kβ in the heart and in the ischemic condition. To clearly elucidate PI3Kα functions, pharmacological approach through PI3Kα inhibitor administration and genetic methods using endothelial- and cardiomyocyte-specific PI3Kα inactivation in mice were used. Exploration of PI3Kβ function was achieved using endothelial- and cardiomyocyte-specific PI3Kβ inactivated mice. Moreover, left anterior descending artery ligation technique was adopted as the MI model because left anterior descending artery is commonly affected in STEMI patient and LAD infarctions are associated with high risk of adverse outcome[291,292]. In addition, human cardiac samples were used to test the changes of PI3K/Akt signaling after MI. I summarize and discuss the major findings in the dissertation as follows.

5.1.1. Administration of PI3Kα Inhibitor-BYL719 Leads to Subtle Cardiac Dysfunction in the Heart and Adverse Ventricular Remodeling after Myocardial Infarction

Despite that a single dose of PI3Kα inhibitor, including BYL719 and TAK117, administration in healthy subjects did not raise any cardiac safety concern in early clinical trial[232,293], I found that mice with 10-day BYL719 treatment developed subtle systolic dysfunction using echocardiographic strain analysis, with normal left ventricular ejection fraction. This is largely due to the higher sensitivity of global longitudinal strain in detecting early subclinical cardiomyopathy than EF[294]. Besides this finding, our results were consistent with previous publications on the regulatory effects of BYL719 on body weight, heart weight, blood glucose, and electrocardiographic alterations[212,214], which might be associated with the inhibitory effects of BYL719 on systemic context. Previous research has highlighted the regulatory role of PI3Kα in energy expenditure, growth, locomotor activity, and thermogenesis, which leads to the disruption of PI3Kα signaling with BYL719 intake, producing a lean phenotype[295]. And insulin/PI3Kα/Akt signaling is essential in the regulation of glucose uptake in skeletal muscles and hepatocytes; thus, BYL719 administration leads to transient hyperglycemia possibly associated with hepatocyte and skeletal myocyte PI3Kα inhibition. Interestingly, with a decrease in muscle mass, there is an increase in fat deposition in BYL719-treated mice. This highlights the differential impacts of BYL719 on different cell types. In the skeletal muscle, IGF/PI3K signaling supports skeletal muscle hypertrophy and blocks muscle atrophy[296]; hence, the use of BYL719 leads to the reduction in skeletal muscle mass. However, the studies on adipose PI3Kα have shown mixed results. Whilst low dose of pan-PI3K inhibitor decreased adiposity in obese mice, loss of PI3Kα specifically in adipose tissue resulted in increased adiposity[297,298]. Our

finding on the increase in fat mass with BYL719 might be associated with the alterations on other signaling pathway by cross-talking with PI3Kα signaling and the systemic feedback regulation after PI3Kα inhibition such as enhanced insulin release[203], resulted in a stimulation of lipogenesis and inhibition of lipolysis. Overall, our findings suggest that systemic use of PI3Kα inhibitor have systemic effects in mice, affecting growth, body composition, and, most importantly, cardiac function. In the clinical setting, cardiac dysfunction, muscle mass reduction, and adiposity are indicators of adverse outcome in various diseases.

When facing MI challenge, PI3Kα inhibition led to exacerbated cardiac dysfunction, increased apoptotic cell death, enhanced inflammation, hypertrophic suppression, and decreased vascular density in the ischemic area. And these effects are accompanied by suppressed Akt/GSK3β and Akt/eNOS signaling. Numerous studies have highlighted the importance of PI3Kα in cell survival and cell growth; however, none of them has investigated the consequence of PI3Kα inhibitors coincided with a heart attack. Preclinical studies implied the therapeutic potential of pharmaceutical PI3Kα inhibitors in atherosclerosis, inflammation, thrombosis, and obesity because of its inhibitory effects on vascular smooth muscle cell proliferation and migration, TNFα-induced vascular permeabilization and leukocyte migration, platelet activation, and body weight[193,234,236,299]. Most importantly, PI3Kα inhibitors, mainly BYL719 (Alpelisib), have been in clinical trials for cancer treatment since 2015, showing efficacy in advanced tumors such as breast cancer, colorectal cancer, and ovarian cancer[206,250,300,301]. Because of the shared biology of cardiovascular disease and cancer, research has shown that cancer patients had an increased rate of CVD, while MI patients showed a higher incidence of cancer[242,246,248,302]. Thus, our studies have highlighted that devastated cardiac outcomes might develop in MI patients who are taking PI3Kα inhibitor medication.

5.1.2. Endothelial PI3Kα is Crucial in Post-MI Vascular Repair by Supporting Angiogenesis

As compared to constitutive lack of endothelial PI3Kα activity mice which developed angiogenic defect during embryonic development[189], loss of endothelial PI3Kα activity during adulthood had no effect on vascular density and cardiac function. This is consistent with the notion that quiescent endothelial cells lining mature vessels are resistant to apoptosis and refractory to VEGF stimulation[303]. Thus, lack of PI3Kα, the major PI3K isoform in the endothelial cells, did not affect PI3K/Akt/eNOS signaling at baseline. However, when experiencing a heart attack, mice without endothelial PI3Kα activity developed severe systolic dysfunction with enhanced inflammation, increased endothelial apoptosis, decreased endothelial proliferation,

and reduced vascular density. *In vitro* experiments on cultured endothelial cells confirmed the importance of PI3Kα in endothelial survival, proliferation, and angiogenesis. These findings are consistent with previous studies highlighting the critical roles of PI3Kα in different endothelial cell types[189]. Importantly, this is the first study, to the best of my knowledge, exploring endothelial PI3Kα in the heart and on how it regulates post-infarct cardiac remodeling. After MI, vascular repairs have been triggered by the means of angiogenesis and bone marrow-derived progenitor cells such as endothelial progenitor cells recruitment, which is associated with the increase in angiogenic signals[81,304,305]. Moreover, enhanced angiogenesis has shown cardioprotective effects via suppressing cardiac remodeling[19]. Surely, cardiac endothelial functions regulate cardiac contractility and cardiac remodeling via paracrine signals such as interleukins, IGF-1, and apelin[306]. In the study, I agree with others that the degree of angiogenesis affects post-MI outcomes; moreover, inhibition of endothelial PI3Kα compromises angiogenic effects, subsequently exacerbating post-MI cardiac function.

5.1.3. Cardiomyocyte PI3Kα Plays Critical Roles in the Healthy and Ischemic Heart

Using genetically modified mice with cardiomyocyte-specific PI3Kα inactivation, I established that PI3Kα regulates cardiac function at baseline and after MI. Mice without cardiomyocyte-PI3Kα developed a mild decrease in systolic function and GSK3β phosphorylation with a slight decreased trend in cardiomyocyte size. The study is partially consistent with previous studies demonstrating the role of PI3Kα/Akt/GSK3β signaling in cardiac contractility and cardiomyocyte size[130,179,307]. Previous studies on different cardiac-specific, genetically modified PI3Kα mice have shown mixed results on cardiomyocyte size and cardiac function, which might be largely attributed to strain differences and various compensatory processes. While constitutively active or dominant negative PI3Kα expression affected cardiomyocyte size without impacts on cardiac function, conditional knockout of cardiac-specific PI3Kα showed a reduction in systolic function due to decreased calcium current[179,180]. Moreover, cardiac-specific and conditional overexpression of PI3Kα has resulted in increased contractility with increased Ca^{2+}-regulating proteins without hypertrophic effect[181]. Overall, our findings on baseline p110α-αMHC mice indicate that cardiac PI3Kα regulates heart size, cardiac contractility, and GSK3β signaling.

Being vital after MI, cardiomyocyte PI3Kα/Akt signaling controls post-MI survival and cardiac function, associated with apoptotic and hypertrophic regulation. Lin et al have demonstrated that cardiac PI3Kα supported post-MI systolic function using mice with increased or decreased PI3Kα activity, and they pointed out the role of PI3Kα in miRNA and mRNA

regulation[188]. Our findings, showing similar preservative effect of PI3Kα on post-MI cardiac function using a different genetic model, highlighting PI3Kα in the regulation of hypoxia induced cardiomyocyte apoptosis and MI triggered hypertrophic effects. *In vivo* and *in vitro* experiments in the study showed that PI3Kα inhibition enhanced MI-associated cardiomyocyte apoptosis with decreased Akt signaling, and this is consistent with previous studies supporting the cardioprotective effects of PI3Kα in dilated cardiomyopathy, doxorubicin-induced cardiomyopathy, diabetic cardiomyopathy, and heart failure models[183,184,187,308]. Using cultured neonatal cardiomyocytes or immortalized cardiomyocytes, research has long shown the enhanced Akt signaling after hypoxic treatment and confirmed the anti-apoptotic effects of PI3K/Akt signaling[259,265,266,309,310]. Back up by these studies, I underlined the importance of PI3Kα isoform in conducting the anti-apoptotic effects of PI3K/Akt/GSK3β signaling using hypoxia-treated primary adult cardiomyocytes and MI model. Moreover, despite current research has highlighted GPCR/G protein signaling in pathological cardiac hypertrophy, PI3K/Akt signaling also contributes to pathological cardiac hypertrophy[311]. The study showed that PI3Kα also participated in post-MI cardiac hypertrophy. In addition, previous research from our research group has shown that PI3Kα/PIP3 controls actin cytoskeleton remodeling by negatively regulating gelsolin[152]. Similar to a study on neonatal rat cardiomyocytes showing enhanced F-actin content after hypoxia[312], I observed an increase in F-/G-actin ratio after hypoxia exposure and PI3Kα inhibitor suppressed this enhancement. These results not only confirm the inhibitory effect of BYL719 in hypoxia-induced PI3Kα activity, but also indicate the role of PI3Kα in hypoxia-mediated actin cytoskeleton remodeling.

5.1.4. PI3Kβ Coordinates with PI3Kα in the Regulation of Endothelial PI3K/Akt/eNOS Signaling

Inactivation of PI3Kβ during adulthood had no effect on cardiac function as previous publication, which is partially related to the quiescent stage of endothelial cells in mature vessels, the dominant effect of PI3Kα in RTK signaling instead of PI3Kβ, and the redundant functions of PI3Kβ with PI3Kγ in GPCR signaling[189,195,216,303]. Surprisingly, mice without endothelial PI3Kβ activity recovered better from MI, with enhanced survival rate, improved systolic function, and decreased disease markers. And this is associated with increased vascular density in the ischemic area and enhanced Akt/eNOS signaling. *In vivo* endothelial cell culture confirmed that PI3Kβ deletion or inhibition enhanced VEGF-induced PI3Kα/Akt/eNOS signaling and angiogenesis. Combined with these findings, I proposed that PI3Kβ activity functions negatively to suppress dominant VEGF/PI3Kα/Akt/eNOS signals in endothelial cells;

thus, suppression of endothelial PI3Kβ enhances MI-triggered angiogenic effects in the heart, leading to better post-MI recovery. Regarding the roles of PI3Kβ in RTK/Akt signaling, mixed results have been shown in the current literature. A study on myoblasts has suggested that the kinase-independent function of PI3Kβ on endocytosis negatively regulation IGF-1/PI3Kα/Akt signaling, while isolated cardiac endothelial cells from mice have shown no effect of lack of PI3Kβ in VEGF/Akt signaling[117,189]. In fibroblasts and primary endothelial progenitor cells, neither the genetic knockout of PI3Kβ activity nor short-term (1-hour) PI3Kβ inhibition has influenced RTK/Akt signaling[94,216]. However, research on PI3K isoform-specific inhibitors has supported the rebound PI3K/Akt signaling with the long-term (more than 6 hours) use of PI3Kβ inhibitor through releasing the inhibitory effect of PI3Kβ on PI3Kα in PTEN-mutated prostate carcinoma cell line[287]. In the dissertation, our findings showed that not only PI3Kβ knockout, but also long-term PI3Kβ inhibition in endothelial cells augmented VEGF/PI3Kα/Akt signaling. These mixed results highlight the potential cell line-specific and time-dependent functions of PI3Kβ inhibitors. Nevertheless, further experiments are required to further elucidate the role of PI3Kβ in the endothelial cells. But importantly, this study has emphasized the potential of targeting endothelial PI3Kβ in MI treatment.

5.1.5. Nuclear PI3Kβ is Essential in Maintaining Cardiomyocyte Survival

Consistent with previous publication[152,214], lack of cardiomyocyte PI3Kβ activity has no effect on cardiac function despite that PI3Kβ was presenting in cardiomyocyte nuclei. However, without cardiomyocyte PI3Kβ, mice developed exacerbated systolic dysfunction and adverse ventricular remodeling after MI, which was associated with increased cardiomyocyte apoptosis. Further analyses showed that cardiomyocyte PI3Kβ activity deficiency reduced nuclear Akt activation and altered gene expression with enhanced expression of genes related to cell death and inflammation, resulting in increased caspases, Bax, Bak, and RIP3 protein levels. PI3Kβ has been previously identified carrying nuclear localization signals, with a nuclear localization signal in p110β C2 domain and a nuclear export sequence in p85β regulatory subunit[111]. Moreover, the nuclear PI3Kβ has been demonstrated to control cell survival, to guild double-strand break repair, and to promote cardiomyocyte proliferation and survival downstream Yes-associated protein[110,111,197]. In addition, nuclear Akt has participated in apoptosis inhibition in different cell types[146,147]. Specifically, it has prevented hypoxia-induced cardiomyocyte apoptosis with changes in the transcriptional profile[290]. In the dissertation, although I could not identify the precise effector between nuclear PI3Kβ and nuclear Akt in the anti-cell death effects and the specific nuclear candidates that nuclear PI3Kβ/Akt targetes, I revealed that lack of PI3Kβ

sensitized hypoxia-related cardiomyocyte death by altering the transcriptional profile and subsequently enhancing cell death protein levels.

5.2. Targeting PI3K Isoforms in Ischemic Heart Disease

Into the 21st century, IHD remains one the leading cause of death worldwide. Knowledge of the mechanisms related to the development and progression of IHD is critical for the development of novel treatments and for a better disease management. The concept that the formation of coronary artery thrombosis as the result of atherosclerotic plaque rupture or erosion triggers MI dominates our understanding of the pathophysiology of MI for decades[313]. Atherosclerosis is a chronic arterial wall inflammatory disease involved a combination of endothelial dysfunction, lipid deposition in the intima, macrophage infiltration, vascular smooth muscle cell proliferation, and extracellular remodeling, resulting in atherosclerotic plaque formation. Upon atherosclerotic plaque rupture or erosion, the exposure of plaque core components and vascular structures rapidly initiate the processes of thrombus formation, including the recruitment of circulating platelets and the activation of coagulation cascade[314]. Subsequently, the formation of an occlusive coronary thrombus leads to MI. In addition, largely resulted from atherosclerosis, MI has been demonstrated to accelerate atherosclerosis associated with increased monocyte recruitment[315]. Thus, besides tackling atherosclerosis, a better management of post-MI cardiac remodeling could yield better clinical outcomes.

In the dissertation, I have investigated the role of PI3Kα and PI3Kβ in post-infarct cardiac remodeling and proposed the potential of manipulating PI3K isoforms in MI treatment. Class I PI3Ks, activated by RTKs and/or GPCRs, engage multiple downstream intracellular effects, importantly Akt, in the regulation of cell survival, growth, migration, proliferation, and metabolism through the production of the lipid second messenger PIP3. The four PI3K isoforms, PI3Kα, PI3Kβ, PI3Kγ, and PI3Kδ, are differentially present in different cell types within the cardiovascular system. During the last decades, the PI3K/Akt pathway has highly impinged in the development of atherosclerosis, thrombosis, and the progression of MI-induced heart failure[316]. Here, I am going to discuss the possibility of targeting PI3K isoforms in atherosclerosis-associated MI.

5.2.1. Manipulating PI3Kα in Ischemic Heart Disease

I have demonstrated the important role of both endothelial- and cardiomyocyte-PI3Kα in post-MI cardiac repair and the devastating results with PI3Kα inhibitor administration after MI. Combined with other findings supporting the importance of PI3Kα in cardiomyocyte survival,

angiogenesis, and post-MI systolic function maintenance[188,189,194,308], amplification of PI3Kα activity has the therapeutic potential to enhance post-MI outcomes. PI3Kα gene therapy has been previously shown to enhance cardiac-specific PI3Kα activity without affecting other organs, and the increase in cardiac PI3Kα activity had no effect on cardiac function at baseline[187]. However, a long-term impact of PI3Kα gene therapy requires a full evaluation before translating to the clinic. Moreover, technological advancement in gene delivery would improve the potential of gene therapy to be a practical tool for treatment.

However, despite the detrimental effect of PI3Kα inhibition in MI, PI3Kα inhibitors have demonstrated inhibitory effects on thrombus formation, suggesting the potential of PI3Kα inhibitors as antiplatelet drugs. Gilio et al have unveiled that PI3Kα inhibition suppressed thrombus formation by reducing glycoprotein VI-dependent calcium responses and downstream platelet aggregation[317]. Moreover, PI3Kα has participated in IGF-1-enhanced platelet activation in an Akt-independent way[299]. However, a study on vascular injury has shown that PI3Kα inhibitor-PIK75 usage had no effect on platelet aggregation, but it inhibited tissue factor and plasminogen activator inhibitor-1 expression and activity and neointima formation by impairing vascular smooth muscle cell proliferation and migration, showing implication in the prevention of injury-induced arterial thrombus formation[236]. In spite of the mixed results and that further investigations are needed to clearly elucidate PI3Kα's involvement in thrombosis, PI3Kα inhibitors have exhibited initial benefits against the pathogenesis of IHD by impairing thrombus formation. However, considering the harmful effect of PI3Kα inhibition in post-MI cardiac remodeling, a more comprehensive knowledge about the effects of systemic administration of PI3Kα inhibitor on atherosclerosis, thrombosis, and post-MI ventricular remodeling as well as the impact of different PI3Kα inhibitor dosages under normal and pathophysiological conditions is needed to weight the benefit and harm of PI3Kα inhibition in IHD. And local use of PI3Kα inhibitor such as via drug-eluting stents might represent a novel therapeutic strategy which potentially yields better clinical outcomes while minimizing unwanted side effects.

In summary, PI3Kα is a therapeutic potential target in IHD treatment. However, extensive experiments further clearing the role of PI3Kα in the development and progression of IHD are dispensable before being translated to the clinical setting. And based on the diverse roles of PI3Kα in different tissue under different medical conditions, it is likely that PI3Kα-targeted therapy would be used in a scenario-based approach.

5.2.2. Manipulating PI3Kβ in Ischemic Heart Disease

During my studies, I was surprised by the distinct role of PI3Kβ in cardiomyocytes and endothelial cells when facing MI. Interestingly, lack of PI3Kβ in the cardiomyocytes sensitized cardiomyocytes to hypoxia induced cell death, causing the detrimental effects under ischemic condition, whilst endothelial cells without PI3Kβ activity was beneficial to the ischemic heart by increasing angiogenesis via PI3Kα/Akt/eNOS signaling. These findings highlight the cell type-specific role of PI3Kβ. On one hand, our results on cardiomyocyte-PI3Kβ are consistent with a previous study which has demonstrated that cardiac-specific amplification of PI3Kβ via gene therapy has protected the heart from MI by reducing cardiomyocyte apoptosis and promoting cardiomyocyte cell cycle activity[197]. On the other hand, I showed that *in vivo* and *in vitro* PI3Kβ inhibition in cardiac endothelial cells enhanced angiogenesis by increasing PI3Kα/Akt/eNOS signaling; however, this is contradicted with a previous study demonstrating that postnatal lack of endothelial PI3Kβ sensitized mice to acute kidney failure after thrombotic microangiopathy injury by impairing angiogenic processes[216]. These inconsistent findings underline the endothelial heterogeneity in structure and function[318]. Importantly, future studies should target specific sites of the vasculature to maximize therapeutic gain in vascular medicine; in the meantime, the awareness of potential distinct impacts on different vascular beds in health and disease should be raised. Nevertheless, endothelial PI3Kβ inhibition has the therapeutic benefit in managing post-infarct cardiac remodeling.

Besides, studies from the last decade have indicated the therapeutic potential of PI3Kβ inhibitors as novel antithrombotic agents. Jackson et al have shown that PI3Kβ inhibitor-TGX-221 displayed rapid and dose-dependent antithrombotic effect; and a low dose of TGX-221 was more effective in preventing the development of occlusive thrombi than aspirin *in vivo*[319]. Mechanically, PI3Kβ participates in platelet activation and aggregation by sustaining integrin αIIbβ3 activation, coordinating with focal adhesion kinases Pyk2, and GSK3-associated thrombus stabilization[120,319-321]. A further investigation has evaluated the efficacy of PI3Kβ inhibitor-AZD6482 as an antiplatelet drug alone and in combination with other antiplatelet agents such as P2Y12 inhibitor and aspirin[322]. However, one preclinical study has disagreed with others and pointed out the potential of increased bleeding with anti-thrombotic TGX-221 doses[323]. Nevertheless, with great antiplatelet potential, early clinical trials have evidenced a mild and generalized antiplatelet effect of AZD6482 with a safety profile and demonstrated the potential of AZD6482 in combination with other antiplatelet agents[322,324]. Thus, PI3Kβ is an attractive antiplatelet target. With the promising antiplatelet effect of PI3Kβ inhibitors and the

close vicinity of the endothelial cell with the blood stream, it is likely that a low dose of PI3Kβ inhibitor intravenous delivery might provide beneficial effects in acute MI patients by preventing further thrombus formation and improving cardiac vascular repair. However, further studies should be conducted on the dose relationship on post-infarct cardiac remodeling.

In summary, despite the damaging effect seen in the lack of cardiomyocyte PI3Kβ activity, the PI3Kβ inhibitor remains an attractive therapeutic potential agent in managing IHD because of its antiplatelet and pro-angiogenic effects. However, thorough investigations on the usage of PI3Kβ inhibitor in the ischemic heart are required to ensure the efficiency and safety of PI3Kβ inhibitors in IHD.

5.3. Concluding Remarks

Altogether, the studies have demonstrated the cell type-specific function of PI3Kα and PI3Kβ in the cardiac tissue in the healthy and ischemic heart. Specifically, PI3Kα is required in cardiomyocytes to maintain cardiac size and function and to fight against hypoxia induced apoptosis, whereas endothelial PI3Kα is critical to maintain endothelial survival, proliferation, and angiogenesis during cardiac injury despite the dispensable role of endothelial PI3Kα during adulthood. PI3Kβ plays a distinct and divergent role in cardiomyocytes and endothelial cells. The lack of PI3Kβ activity sensitizes cardiomyocyte to hypoxia induced cell death by enhancing the expression of cell death-associated proteins; on the contrary, PI3Kβ inactivation in endothelial cells promotes post-MI cardiac repair by improving angiogenesis. Therefore, PI3Kα and PI3Kβ are nonredundant in cardiac tissue to maintain proper cardiac function and to regulate post-infarct cardiac repair.

Manipulation of PI3Kα and/or PI3Kβ has therapeutic potential in IHD treatment. While amplification of cardiac PI3Kα might provide beneficial effects to the ischemic heart by protecting cardiomyocyte from apoptosis and supporting angiogenesis, small molecules drug targeting PI3Kβ might improves post-MI cardiac repair by enhancing vascular repair and preventing further thrombus formation with its antiplatelet effects. Moreover, with the growing evidence supporting the use of PI3Kα inhibitors in advanced tumors, the concerns of potential cardiotoxicity of chronic treatments should be raised, especially when coinciding with an ischemic event. Therefore, efforts should be undertaken to clarify the functions of PI3Kα and PI3Kβ in the heart that could enhance the therapeutic index of targeting these isoforms in IHD treatment and minimize the cardiac side effects when systemic administration of drugs targeting PI3Kα or PI3Kβ.

Chapter 6

Limitations and Future Directions

6.1. Limitations

6.1.1. Genetically Modified Mice

The Cre mouse models in the dissertation are powerful tools that allow me to study specific PI3K isoform using mice with cell type-specific genetic loss of function; however, there are limitations. Studying the role of PI3K isoform in endothelial cells, the Tie2-MerCreMer system was used to generate inducible endothelial genetic loss of function, which showed no effect on the hematopoietic lineage when giving tamoxifen to the adult mice. However, commonly used endothelial-specific Cre models, including the *Tie2*-Cre system and VE-Cadherin-Cre system, have been shown to affect lymphatic vasculature as well[325], making it impossible to separate between the effects in vascular beds and in the lymphatic vasculature. When studying the functions of PI3K isoforms in the cardiomyocytes, genetically modified mice with gene deletion specifically in cardiomyocyte was driven by α-MHC promoter which is commonly used and provides robust expression in ventricular cardiomyocyte after birth[326]. Without using inducible Cre activation, the effects of loss of PI3K isoform activity during embryonic development cannot be completely ruled out; and the compensatory effects due to the loss of PI3K isoform activity might also participate in the outcomes.

6.1.2. Experimental Myocardial Infarction Model

The left anterior descending artery ligation procedure is one of the most widely used models of MI in mice. In the dissertation, all mice were randomly assigned to receive MI or sham surgery, and the surgery was performed by a technician who was blind to the genotypes, making our results more reliable and reproducible. However, the development and progression of MI are affected by genetic and environmental factors and comorbidity in the clinical environment. The adopted MI model in the dissertation was carried out in young adult mice without any cardiovascular risk factors; therefore, future studies on aged, spontaneous atherosclerotic plaque developed, obese, and/or high-fat diet mice should be performed. In addition, because of the difference in disease development and progression in male and female, only male mice were used in current study to pinpoint the role of PI3K isoforms in post-MI cardiac remodeling. Aged female mice should be used to study the PI3K isoform functions as well.

6.2. Future Directions

6.2.1. To Further Study the Long-term Effects of PI3Kα Inhibitor Usage and the Potential of Heart Function Recovery after Drug Withdrawal

During my graduate studies, I found that 10-day PI3Kα inhibitor administration led to subtle systolic dysfunction and an MI event during drug administration worsened post-MI outcomes. With the use of PI3Kα inhibitors in cancer treatment which will require long-term usage of PI3Kα inhibitors, these findings raise questions: (i) does long-term PI3Kα inhibition influence cardiac function; (ii) will the heart recovery from the effects of short-term or long-term PI3Kα inhibitor usage; (iii) will discontinuance of PI3Kα inhibitor usage when experiencing a heart attack impact post-MI outcomes. Further investigation of these questions will provide better guidance on PI3Kα usage in high-risk patients.

6.2.2. To Investigate the Impacts of PI3Kβ Inhibitor on the Heart and the Potential of Using low-dose PI3Kβ Inhibitor in MI Treatment

Since PI3Kβ inhibitors, such as GSK2636771 and AZD6482, have been used in clinical trials tackling PTEN-deficient advanced tumor and thrombosis specifically and inhibition of endothelial PI3Kβ displayed cardioprotective effects after MI, there is potential of using low-dose PI3Kβ inhibitor in MI treatment by preventing further thrombus formation and supporting post-MI angiogenesis. Thus, further studies on the following questions will explore the therapeutic potential of PI3Kβ inhibitors in MI: (i) the impact of difference doses (e.g. low-dose or high-dose) of PI3Kβ inhibitor administration on cardiac function; (ii) and will the use of low-dose or high-dose PI3Kβ inhibitor upon MI influence post-MI outcomes.

6.2.3. To Further Explore the Systemic Effects of PI3Kα and the Role of PI3Kα in the Adipose Tissue and Skeletal Muscles

In the study, I have shown that PI3Kα inhibitor usage was associated with a marked increase in fat mass and a significant decrease in lean mass, which brings up the question how BYL719 administration affects body composition. With clinical studies highlighting the importance of fat mass and lean mass in the prognosis of cardiovascular disease and chronic disease, it is critical to explore the effect of PI3Kα inhibitor administration in body composition alteration. Therefore, studies in the future investigate the following questions should be considered: (i) how the administration of PI3Kα inhibitor alters composition and molecular signaling in adipose tissue and skeletal muscles; (ii) what the function of PI3Kα is in adipose tissue; (iii) how PI3Kα regulates muscle mass.

References

1   Roth, G. A. *et al.* Global, Regional, and National Burden of Cardiovascular Diseases for 10 Causes, 1990 to 2015. *Journal of the American College of Cardiology* **70**, 1-25, doi:10.1016/j.jacc.2017.04.052 (2017).

2   Global, regional, and national age-sex specific mortality for 264 causes of death, 1980-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet (London, England)* **390**, 1151-1210, doi:10.1016/s0140-6736(17)32152-9 (2017).

3   Benjamin, E. J. *et al.* Heart Disease and Stroke Statistics-2019 Update: A Report From the American Heart Association. *Circulation* **139**, e56-e528, doi:10.1161/cir.0000000000000659 (2019).

4   Roth, G. A. *et al.* The Burden of Cardiovascular Diseases Among US States, 1990-2016. *JAMA cardiology* **3**, 375-389, doi:10.1001/jamacardio.2018.0385 (2018).

5   Mendis, S. *et al.* World Health Organization definition of myocardial infarction: 2008-09 revision. *International journal of epidemiology* **40**, 139-146, doi:10.1093/ije/dyq165 (2011).

6   Anderson, J. L. & Morrow, D. A. Acute Myocardial Infarction. *The New England journal of medicine* **376**, 2053-2064, doi:10.1056/NEJMra1606915 (2017).

7   Mozaffarian, D. *et al.* Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. *Circulation* **133**, e38-360, doi:10.1161/cir.0000000000000350 (2016).

8   Yeh, R. W. *et al.* Population trends in the incidence and outcomes of acute myocardial infarction. *The New England journal of medicine* **362**, 2155-2165, doi:10.1056/NEJMoa0908610 (2010).

9   Takii, T. *et al.* Trends in acute myocardial infarction incidence and mortality over 30 years in Japan: report from the MIYAGI-AMI Registry Study. *Circulation journal : official journal of the Japanese Circulation Society* **74**, 93-100 (2010).

10   Turk-Adawi, K. *et al.* Cardiovascular disease in the Eastern Mediterranean region: epidemiology and risk factor burden. *Nature reviews. Cardiology* **15**, 106-119, doi:10.1038/nrcardio.2017.138 (2018).

11   Sulo, G. *et al.* Heart Failure Complicating Acute Myocardial Infarction; Burden and Timing of Occurrence: A Nation-wide Analysis Including 86 771 Patients From the Cardiovascular Disease in Norway (CVDNOR) Project. *Journal of the American Heart Association* **5**, doi:10.1161/jaha.115.002667 (2016).

12   Gabriel-Costa, D. The pathophysiology of myocardial infarction-induced heart failure. *Pathophysiology : the official journal of the International Society for Pathophysiology*, doi:10.1016/j.pathophys.2018.04.003 (2018).

13   Sutton, M. G. & Sharpe, N. Left ventricular remodeling after myocardial infarction: pathophysiology and therapy. *Circulation* **101**, 2981-2988 (2000).

14   Gajarsa, J. J. & Kloner, R. A. Left ventricular remodeling in the post-infarction heart: a review of cellular, molecular mechanisms, and therapeutic modalities. *Heart failure reviews* **16**, 13-21, doi:10.1007/s10741-010-9181-7 (2011).

15   Chiong, M. *et al.* Cardiomyocyte death: mechanisms and translational implications. *Cell death & disease* **2**, e244, doi:10.1038/cddis.2011.130 (2011).

16   de Haan, J. J., Smeets, M. B., Pasterkamp, G. & Arslan, F. Danger signals in the initiation of the inflammatory response after myocardial infarction. *Mediators of inflammation* **2013**, 206039, doi:10.1155/2013/206039 (2013).

17   Arandjelovic, S. & Ravichandran, K. S. A MERry response after myocardial infarction. *Circulation research* **113**, 949-951, doi:10.1161/circresaha.113.302155 (2013).

18   Nakaya, M. *et al.* Cardiac myofibroblast engulfment of dead cells facilitates recovery after myocardial infarction. *The Journal of clinical investigation* **127**, 383-401, doi:10.1172/jci83822 (2017).

19   Kobayashi, K. *et al.* Dynamics of angiogenesis in ischemic areas of the infarcted heart. *Scientific reports* **7**, 7156, doi:10.1038/s41598-017-07524-x (2017).

20   Cochain, C., Channon, K. M. & Silvestre, J. S. Angiogenesis in the infarcted myocardium. *Antioxidants & redox signaling* **18**, 1100-1113, doi:10.1089/ars.2012.4849 (2013).

21   Shiojima, I. *et al.* Disruption of coordinated cardiac hypertrophy and angiogenesis contributes to the transition to heart failure. *The Journal of clinical investigation* **115**, 2108-2118, doi:10.1172/jci24682 (2005).

22   Niu, J. *et al.* Correlation between vascular endothelial growth factor and long-term prognosis in patients with acute myocardial infarction. *Experimental and therapeutic medicine* **12**, 475-479, doi:10.3892/etm.2016.3286 (2016).

23   Abdurrachim, D. *et al.* Good and bad consequences of altered fatty acid metabolism in heart failure: evidence from mouse models. *Cardiovascular research* **106**, 194-205, doi:10.1093/cvr/cvv105 (2015).

24   Bhar-Amato, J., Davies, W. & Agarwal, S. Ventricular Arrhythmia after Acute Myocardial Infarction: 'The Perfect Storm'. *Arrhythmia & electrophysiology review* **6**, 134-139, doi:10.15420/aer.2017.24.1 (2017).

25   Thum, T. *et al.* Bone marrow molecular alterations after myocardial infarction: Impact on endothelial progenitor cells. *Cardiovascular research* **70**, 50-60, doi:10.1016/j.cardiores.2006.01.002 (2006).

26   Fang, L., Moore, X. L., Dart, A. M. & Wang, L. M. Systemic inflammatory response following acute myocardial infarction. *Journal of geriatric cardiology : JGC* **12**, 305-312, doi:10.11909/j.issn.1671-5411.2015.03.020 (2015).

27   Zimmermann, M. *et al.* Analysis of region specific gene expression patterns in the heart and systemic responses after experimental myocardial ischemia. *Oncotarget* **8**, 60809-60825, doi:10.18632/oncotarget.17955 (2017).

28   Bergmann, O. *et al.* Dynamics of Cell Generation and Turnover in the Human Heart. *Cell* **161**, 1566-1575, doi:10.1016/j.cell.2015.05.026 (2015).

29   Pinto, A. R. *et al.* Revisiting Cardiac Cellular Composition. *Circulation research* **118**, 400-409, doi:10.1161/circresaha.115.307778 (2016).

30   Tirziu, D., Giordano, F. J. & Simons, M. Cell communications in the heart. *Circulation* **122**, 928-937, doi:10.1161/circulationaha.108.847731 (2010).

31   Frangogiannis, N. G. Pathophysiology of Myocardial Infarction. *Comprehensive Physiology* **5**, 1841-1875, doi:10.1002/cphy.c150006 (2015).

32   Algranati, D., Kassab, G. S. & Lanir, Y. Why is the subendocardium more vulnerable to ischemia? A new paradigm. *American journal of physiology. Heart and circulatory physiology* **300**, H1090-1100, doi:10.1152/ajpheart.00473.2010 (2011).

33   Kalogeris, T., Baines, C. P., Krenz, M. & Korthuis, R. J. Ischemia/Reperfusion. *Comprehensive Physiology* **7**, 113-170, doi:10.1002/cphy.c160006 (2016).

34   Kostin, S. *et al.* Myocytes die by multiple mechanisms in failing human hearts. *Circulation research* **92**, 715-724, doi:10.1161/01.res.0000067471.95890.5c (2003).

35   Amgalan, D., Chen, Y. & Kitsis, R. N. Death Receptor Signaling in the Heart: Cell Survival, Apoptosis, and Necroptosis. *Circulation* **136**, 743-746, doi:10.1161/circulationaha.117.029566 (2017).

36   Konstantinidis, K., Whelan, R. S. & Kitsis, R. N. Mechanisms of cell death in heart disease. *Arteriosclerosis, thrombosis, and vascular biology* **32**, 1552-1562, doi:10.1161/atvbaha.111.224915 (2012).

37   Kanamori, H. *et al.* Autophagy limits acute myocardial infarction induced by permanent coronary artery occlusion. *American journal of physiology. Heart and circulatory physiology* **300**, H2261-2271, doi:10.1152/ajpheart.01056.2010 (2011).

38   Matsui, Y. *et al.* Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy. *Circulation research* **100**, 914-922, doi:10.1161/01.res.0000261924.76669.36 (2007).

39   Maiuri, M. C., Zalckvar, E., Kimchi, A. & Kroemer, G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nature reviews. Molecular cell biology* **8**, 741-752, doi:10.1038/nrm2239 (2007).

40   Nishida, K., Yamaguchi, O. & Otsu, K. Crosstalk between autophagy and apoptosis in heart disease. *Circulation research* **103**, 343-351, doi:10.1161/circresaha.108.175448 (2008).

41   Knaapen, M. W. *et al.* Apoptotic versus autophagic cell death in heart failure. *Cardiovascular research* **51**, 304-312, doi:10.1016/s0008-6363(01)00290-5 (2001).

42   Stiermaier, T. *et al.* Impact of left ventricular hypertrophy on myocardial injury in patients with ST-segment elevation myocardial infarction. *Clinical research in cardiology : official journal of the German Cardiac Society* **107**, 1013-1020, doi:10.1007/s00392-018-1273-8 (2018).

43   Nepper-Christensen, L. *et al.* Left Ventricular Hypertrophy Is Associated With Increased Infarct Size and Decreased Myocardial Salvage in Patients With ST-Segment Elevation Myocardial Infarction Undergoing Primary Percutaneous Coronary Intervention. *Journal of the American Heart Association* **6**, doi:10.1161/jaha.116.004823 (2017).

44   Gidh-Jain, M., Huang, B., Jain, P., Gick, G. & El-Sherif, N. Alterations in cardiac gene expression during ventricular remodeling following experimental myocardial infarction. *Journal of molecular and cellular cardiology* **30**, 627-637, doi:10.1006/jmcc.1997.0628 (1998).

45   Lou, Q., Janardhan, A. & Efimov, I. R. Remodeling of calcium handling in human heart failure. *Advances in experimental medicine and biology* **740**, 1145-1174, doi:10.1007/978-94-007-2888-2_52 (2012).

46   Zhu, Y. C. *et al.* Role of angiotensin AT1 and AT2 receptors in cardiac hypertrophy and cardiac remodelling. *Clinical and experimental pharmacology & physiology* **30**, 911-918 (2003).

47   Archer, C. R., Robinson, E. L., Drawnel, F. M. & Roderick, H. L. Endothelin-1 promotes hypertrophic remodelling of cardiac myocytes by activating sustained signalling and

transcription downstream of endothelin type A receptors. *Cellular signalling* **36**, 240-254, doi:10.1016/j.cellsig.2017.04.010 (2017).

48   Tilley, D. G. G protein-dependent and G protein-independent signaling pathways and their impact on cardiac function. *Circulation research* **109**, 217-230, doi:10.1161/circresaha.110.231225 (2011).

49   Salazar, N. C., Chen, J. & Rockman, H. A. Cardiac GPCRs: GPCR signaling in healthy and failing hearts. *Biochimica et biophysica acta* **1768**, 1006-1018, doi:10.1016/j.bbamem.2007.02.010 (2007).

50   Diviani, D., Maric, D., Perez Lopez, I., Cavin, S. & Del Vescovo, C. D. A-kinase anchoring proteins: molecular regulators of the cardiac stress response. *Biochimica et biophysica acta* **1833**, 901-908, doi:10.1016/j.bbamcr.2012.07.014 (2013).

51   Molkentin, J. D. Calcineurin-NFAT signaling regulates the cardiac hypertrophic response in coordination with the MAPKs. *Cardiovascular research* **63**, 467-475, doi:10.1016/j.cardiores.2004.01.021 (2004).

52   Palaniyandi, S. S., Sun, L., Ferreira, J. C. & Mochly-Rosen, D. Protein kinase C in heart failure: a therapeutic target? *Cardiovascular research* **82**, 229-239, doi:10.1093/cvr/cvp001 (2009).

53   Vega, R. B. *et al.* Protein kinases C and D mediate agonist-dependent cardiac hypertrophy through nuclear export of histone deacetylase 5. *Molecular and cellular biology* **24**, 8374-8385, doi:10.1128/mcb.24.19.8374-8385.2004 (2004).

54   Braz, J. C., Bueno, O. F., De Windt, L. J. & Molkentin, J. D. PKC alpha regulates the hypertrophic growth of cardiomyocytes through extracellular signal-regulated kinase1/2 (ERK1/2). *The Journal of cell biology* **156**, 905-919, doi:10.1083/jcb.200108062 (2002).

55   Wilkins, B. J. & Molkentin, J. D. Calcium-calcineurin signaling in the regulation of cardiac hypertrophy. *Biochemical and biophysical research communications* **322**, 1178-1191, doi:10.1016/j.bbrc.2004.07.121 (2004).

56   Anderson, M. E., Brown, J. H. & Bers, D. M. CaMKII in myocardial hypertrophy and heart failure. *Journal of molecular and cellular cardiology* **51**, 468-473, doi:10.1016/j.yjmcc.2011.01.012 (2011).

57   Li, B., Dedman, J. R. & Kaetzel, M. A. Nuclear Ca2+/calmodulin-dependent protein kinase II in the murine heart. *Biochimica et biophysica acta* **1763**, 1275-1281, doi:10.1016/j.bbamcr.2006.09.029 (2006).

58   Morel, E. *et al.* cAMP-binding protein Epac induces cardiomyocyte hypertrophy. *Circulation research* **97**, 1296-1304, doi:10.1161/01.res.0000194325.31359.86 (2005).

59   Metrich, M. *et al.* Epac mediates beta-adrenergic receptor-induced cardiomyocyte hypertrophy. *Circulation research* **102**, 959-965, doi:10.1161/circresaha.107.164947 (2008).

60   Rysa, J., Tokola, H. & Ruskoaho, H. Mechanical stretch induced transcriptomic profiles in cardiac myocytes. *Scientific reports* **8**, 4733, doi:10.1038/s41598-018-23042-w (2018).

61   Harston, R. K. & Kuppuswamy, D. Integrins are the necessary links to hypertrophic growth in cardiomyocytes. *Journal of signal transduction* **2011**, 521742, doi:10.1155/2011/521742 (2011).

62   Israeli-Rosenberg, S., Manso, A. M., Okada, H. & Ross, R. S. Integrins and integrin-associated proteins in the cardiac myocyte. *Circulation research* **114**, 572-586, doi:10.1161/circresaha.114.301275 (2014).

63   Fredj, S. *et al.* Role of interleukin-6 in cardiomyocyte/cardiac fibroblast interactions during myocyte hypertrophy and fibroblast proliferation. *Journal of cellular physiology* **204**, 428-436, doi:10.1002/jcp.20307 (2005).

64   Palmen, M. *et al.* Cardiac remodeling after myocardial infarction is impaired in IGF-1 deficient mice. *Cardiovascular research* **50**, 516-524, doi:10.1016/s0008-6363(01)00237-1 (2001).

65   Matsumoto-Ida, M. *et al.* Activation of TGF-beta1-TAK1-p38 MAPK pathway in spared cardiomyocytes is involved in left ventricular remodeling after myocardial infarction in rats. *American journal of physiology. Heart and circulatory physiology* **290**, H709-715, doi:10.1152/ajpheart.00186.2005 (2006).

66   Zhao, L. *et al.* Deletion of Interleukin-6 Attenuates Pressure Overload-Induced Left Ventricular Hypertrophy and Dysfunction. *Circulation research* **118**, 1918-1929, doi:10.1161/circresaha.116.308688 (2016).

67   Yamaguchi, K. *et al.* Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. *Science (New York, N.Y.)* **270**, 2008-2011, doi:10.1126/science.270.5244.2008 (1995).

68   Nishikimi, T., Maeda, N. & Matsuoka, H. The role of natriuretic peptides in cardioprotection. *Cardiovascular research* **69**, 318-328, doi:10.1016/j.cardiores.2005.10.001 (2006).

69   Kosutova, M., Pechanova, O., Barta, A., Franova, S. & Cebova, M. Different adaptive NO-dependent Mechanisms in Normal and Hypertensive Conditions. *Molecules (Basel, Switzerland)* **24**, doi:10.3390/molecules24091682 (2019).

70   Fraccarollo, D. *et al.* Improvement in left ventricular remodeling by the endothelial nitric oxide synthase enhancer AVE9488 after experimental myocardial infarction. *Circulation* **118**, 818-827, doi:10.1161/circulationaha.107.717702 (2008).

71   Merkus, D. *et al.* Coronary vasoconstrictor influence of angiotensin II is reduced in remodeled myocardium after myocardial infarction. *American journal of physiology. Heart and circulatory physiology* **291**, H2082-2089, doi:10.1152/ajpheart.00861.2005 (2006).

72   de Beer, V. J. *et al.* Prostanoids suppress the coronary vasoconstrictor influence of endothelin after myocardial infarction. *American journal of physiology. Heart and circulatory physiology* **301**, H1080-1089, doi:10.1152/ajpheart.01307.2010 (2011).

73   Kido, M. *et al.* Hypoxia-inducible factor 1-alpha reduces infarction and attenuates progression of cardiac dysfunction after myocardial infarction in the mouse. *Journal of the American College of Cardiology* **46**, 2116-2124, doi:10.1016/j.jacc.2005.08.045 (2005).

74   Pugh, C. W. & Ratcliffe, P. J. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nature medicine* **9**, 677-684, doi:10.1038/nm0603-677 (2003).

75   Ferraro, B. *et al.* Pro-Angiogenic Macrophage Phenotype to Promote Myocardial Repair. *Journal of the American College of Cardiology* **73**, 2990-3002, doi:10.1016/j.jacc.2019.03.503 (2019).

76   Mouton, A. J. *et al.* Fibroblast polarization over the myocardial infarction time continuum shifts roles from inflammation to angiogenesis. *Basic research in cardiology* **114**, 6, doi:10.1007/s00395-019-0715-4 (2019).

77   Kim, C. H., Cho, Y. S., Chun, Y. S., Park, J. W. & Kim, M. S. Early expression of myocardial HIF-1alpha in response to mechanical stresses: regulation by stretch-activated channels and the phosphatidylinositol 3-kinase signaling pathway. *Circulation research* **90**, E25-33, doi:10.1161/hh0202.104923 (2002).

78   Qipshidze, N., Metreveli, N., Mishra, P. K., Lominadze, D. & Tyagi, S. C. Hydrogen sulfide mitigates cardiac remodeling during myocardial infarction via improvement of angiogenesis. *International journal of biological sciences* **8**, 430-441, doi:10.7150/ijbs.3632 (2012).

79   Ogawa, H. *et al.* Increased blood vascular endothelial growth factor levels in patients with acute myocardial infarction. *Cardiology* **93**, 93-99, doi:10.1159/000007008 (2000).

80   Holderfield, M. T. & Hughes, C. C. Crosstalk between vascular endothelial growth factor, notch, and transforming growth factor-beta in vascular morphogenesis. *Circulation research* **102**, 637-652, doi:10.1161/circresaha.107.167171 (2008).

81   Ii, M. *et al.* Endothelial progenitor cells are rapidly recruited to myocardium and mediate protective effect of ischemic preconditioning via "imported" nitric oxide synthase activity. *Circulation* **111**, 1114-1120, doi:10.1161/01.cir.0000157144.24888.7e (2005).

82   Asahara, T. *et al.* Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circulation research* **85**, 221-228, doi:10.1161/01.res.85.3.221 (1999).

83   Tang, Y. L. *et al.* Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction. *The Annals of thoracic surgery* **80**, 229-236; discussion 236-227, doi:10.1016/j.athoracsur.2005.02.072 (2005).

84   Chung, E. S., Packer, M., Lo, K. H., Fasanmade, A. A. & Willerson, J. T. Randomized, double-blind, placebo-controlled, pilot trial of infliximab, a chimeric monoclonal antibody to tumor necrosis factor-alpha, in patients with moderate-to-severe heart failure: results of the anti-TNF Therapy Against Congestive Heart Failure (ATTACH) trial. *Circulation* **107**, 3133-3140, doi:10.1161/01.cir.0000077913.60364.d2 (2003).

85   Hartman, M. H. T., Groot, H. E., Leach, I. M., Karper, J. C. & van der Harst, P. Translational overview of cytokine inhibition in acute myocardial infarction and chronic heart failure. *Trends in cardiovascular medicine* **28**, 369-379, doi:10.1016/j.tcm.2018.02.003 (2018).

86   Rahman, F. A. *et al.* Efficacy and Safety of Cyclosporine in Acute Myocardial Infarction: A Systematic Review and Meta-Analysis. *Frontiers in pharmacology* **9**, 238, doi:10.3389/fphar.2018.00238 (2018).

87   Yla-Herttuala, S., Bridges, C., Katz, M. G. & Korpisalo, P. Angiogenic gene therapy in cardiovascular diseases: dream or vision? *European heart journal* **38**, 1365-1371, doi:10.1093/eurheartj/ehw547 (2017).

88   Yuan, R. *et al.* Vascular endothelial growth factor gene transfer therapy for coronary artery disease: A systematic review and meta-analysis. *Cardiovascular therapeutics* **36**, e12461, doi:10.1111/1755-5922.12461 (2018).

89   Gyongyosi, M., Haller, P. M., Blake, D. J. & Martin Rendon, E. Meta-Analysis of Cell Therapy Studies in Heart Failure and Acute Myocardial Infarction. *Circulation research* **123**, 301-308, doi:10.1161/circresaha.117.311302 (2018).

90   Lalu, M. M. *et al.* Safety and Efficacy of Adult Stem Cell Therapy for Acute Myocardial Infarction and Ischemic Heart Failure (SafeCell Heart): A Systematic Review and Meta-Analysis. *Stem cells translational medicine* **7**, 857-866, doi:10.1002/sctm.18-0120 (2018).

91   Marques, M. *et al.* Phosphoinositide 3-kinases p110alpha and p110beta regulate cell cycle entry, exhibiting distinct activation kinetics in G1 phase. *Molecular and cellular biology* **28**, 2803-2814, doi:10.1128/mcb.01786-07 (2008).

92   Yu, J. S. & Cui, W. Proliferation, survival and metabolism: the role of PI3K/AKT/mTOR signalling in pluripotency and cell fate determination. *Development (Cambridge, England)* **143**, 3050-3060, doi:10.1242/dev.137075 (2016).

93   Hu, H. *et al.* Phosphoinositide 3-Kinase Regulates Glycolysis through Mobilization of Aldolase from the Actin Cytoskeleton. *Cell* **164**, 433-446, doi:10.1016/j.cell.2015.12.042 (2016).

94   Guillermet-Guibert, J. *et al.* The p110β isoform of phosphoinositide 3-kinase signals downstream of G protein-coupled receptors and is functionally redundant with p110γ. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 8292-8297, doi:10.1073/pnas.0707761105 (2008).

95   Knight, Z. A. *et al.* A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling. *Cell* **125**, 733-747, doi:10.1016/j.cell.2006.03.035 (2006).

96   Yang, H. W. *et al.* Cooperative activation of PI3K by Ras and Rho family small GTPases. *Molecular cell* **47**, 281-290, doi:10.1016/j.molcel.2012.05.007 (2012).

97   Castellano, E. *et al.* Requirement for interaction of PI3-kinase p110alpha with RAS in lung tumor maintenance. *Cancer cell* **24**, 617-630, doi:10.1016/j.ccr.2013.09.012 (2013).

98   Pacold, M. E. *et al.* Crystal structure and functional analysis of Ras binding to its effector phosphoinositide 3-kinase gamma. *Cell* **103**, 931-943, doi:10.1016/s0092-8674(00)00196-3 (2000).

99   Fritsch, R. *et al.* RAS and RHO families of GTPases directly regulate distinct phosphoinositide 3-kinase isoforms. *Cell* **153**, 1050-1063, doi:10.1016/j.cell.2013.04.031 (2013).

100   Bi, L., Okabe, I., Bernard, D. J., Wynshaw-Boris, A. & Nussbaum, R. L. Proliferative defect and embryonic lethality in mice homozygous for a deletion in the p110alpha subunit of phosphoinositide 3-kinase. *The Journal of biological chemistry* **274**, 10963-10968, doi:10.1074/jbc.274.16.10963 (1999).

101 Zhao, J. J. *et al.* The p110alpha isoform of PI3K is essential for proper growth factor signaling and oncogenic transformation. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 16296-16300, doi:10.1073/pnas.0607899103 (2006).

102 Bi, L., Okabe, I., Bernard, D. J. & Nussbaum, R. L. Early embryonic lethality in mice deficient in the p110beta catalytic subunit of PI 3-kinase. *Mammalian genome : official journal of the International Mammalian Genome Society* **13**, 169-172, doi:10.1007/s00335-001-2123-x (2002).

103 Hirsch, E. *et al.* Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science (New York, N.Y.)* **287**, 1049-1053, doi:10.1126/science.287.5455.1049 (2000).

104 Clayton, E. *et al.* A crucial role for the p110delta subunit of phosphatidylinositol 3-kinase in B cell development and activation. *The Journal of experimental medicine* **196**, 753-763, doi:10.1084/jem.20020805 (2002).

105 Ciraolo, E. *et al.* Phosphoinositide 3-kinase p110beta activity: key role in metabolism and mammary gland cancer but not development. *Science Signaling* **1**, 1-12 (2008).

106 Sopasakis, V. R. *et al.* Specific roles of the p110alpha isoform of phosphatidylinsositol 3-kinase in hepatic insulin signaling and metabolic regulation. *Cell metabolism* **11**, 220-230, doi:10.1016/j.cmet.2010.02.002 (2010).

107 Dbouk, H. A., Pang, H., Fiser, A. & Backer, J. M. A biochemical mechanism for the oncogenic potential of the p110beta catalytic subunit of phosphoinositide 3-kinase. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 19897-19902, doi:10.1073/pnas.1008739107 (2010).

108 Utermark, T. *et al.* The p110alpha and p110beta isoforms of PI3K play divergent roles in mammary gland development and tumorigenesis. *Genes & development* **26**, 1573-1586, doi:10.1101/gad.191973.112 (2012).

109 Marques, M. *et al.* Specific function of phosphoinositide 3-kinase beta in the control of DNA replication. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 7525-7530, doi:10.1073/pnas.0812000106 (2009).

110 Kumar, A., Fernandez-Capetillo, O. & Carrera, A. C. Nuclear phosphoinositide 3-kinase beta controls double-strand break DNA repair. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 7491-7496, doi:10.1073/pnas.0914242107 (2010).

111   Kumar, A. *et al.* Nuclear but not cytosolic phosphoinositide 3-kinase beta has an essential function in cell survival. *Molecular and cellular biology* **31**, 2122-2133, doi:10.1128/MCB.01313-10 (2011).

112   Thorpe, L. M., Yuzugullu, H. & Zhao, J. J. PI3K in cancer: divergent roles of isoforms, modes of activation and therapeutic targeting. *Nature reviews. Cancer* **15**, 7-24, doi:10.1038/nrc3860 (2015).

113   Jia, S. *et al.* Essential roles of PI(3)K-p110beta in cell growth, metabolism and tumorigenesis. *Nature* **454**, 776-779, doi:10.1038/nature07091 (2008).

114   Dbouk, H. A. *et al.* G protein-coupled receptor-mediated activation of p110beta by Gbetagamma is required for cellular transformation and invasiveness. *Science signaling* **5**, ra89, doi:10.1126/scisignal.2003264 (2012).

115   Gomes, L. C. *et al.* Advances in chronic lymphocytic leukemia pharmacotherapy. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* **97**, 349-358, doi:10.1016/j.biopha.2017.10.105 (2018).

116   Mato, A., Jauhari, S. & Schuster, S. J. Management of chronic lymphocytic leukemia (CLL) in the era of B-cell receptor signal transduction inhibitors. *American journal of hematology* **90**, 657-664, doi:10.1002/ajh.24021 (2015).

117   Matheny, R. W., Jr. & Adamo, M. L. PI3K p110 alpha and p110 beta have differential effects on Akt activation and protection against oxidative stress-induced apoptosis in myoblasts. *Cell death and differentiation* **17**, 677-688, doi:10.1038/cdd.2009.150 (2010).

118   Redondo-Munoz, J., Perez-Garcia, V., Rodriguez, M. J., Valpuesta, J. M. & Carrera, A. C. Phosphoinositide 3-kinase beta protects nuclear envelope integrity by controlling RCC1 localization and Ran activity. *Molecular and cellular biology* **35**, 249-263, doi:10.1128/MCB.01184-14 (2015).

119   Patrucco, E. *et al.* PI3Kgamma modulates the cardiac response to chronic pressure overload by distinct kinase-dependent and -independent effects. *Cell* **118**, 375-387, doi:10.1016/j.cell.2004.07.017 (2004).

120   Cosemans, J. M. *et al.* Continuous signaling via PI3K isoforms beta and gamma is required for platelet ADP receptor function in dynamic thrombus stabilization. *Blood* **108**, 3045-3052, doi:10.1182/blood-2006-03-006338 (2006).

121   Shiojima, I. & Walsh, K. Regulation of cardiac growth and coronary angiogenesis by the Akt/PKB signaling pathway. *Genes & development* **20**, 3347-3365, doi:10.1101/gad.1492806 (2006).

122   Song, G., Ouyang, G. & Bao, S. The activation of Akt/PKB signaling pathway and cell survival. *Journal of cellular and molecular medicine* **9**, 59-71, doi:10.1111/j.1582-4934.2005.tb00337.x (2005).

123   Wang, X. T. *et al.* Opposing effects of Bad phosphorylation at two distinct sites by Akt1 and JNK1/2 on ischemic brain injury. *Cellular signalling* **19**, 1844-1856, doi:10.1016/j.cellsig.2007.04.005 (2007).

124   Pugazhenthi, S. *et al.* Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. *The Journal of biological chemistry* **275**, 10761-10766, doi:10.1074/jbc.275.15.10761 (2000).

125   Shimamura, H. *et al.* The PI3-kinase-Akt pathway promotes mesangial cell survival and inhibits apoptosis in vitro via NF-kappa B and Bad. *Journal of the American Society of Nephrology : JASN* **14**, 1427-1434, doi:10.1097/01.asn.0000066140.99610.32 (2003).

126   Zhang, X., Tang, N., Hadden, T. J. & Rishi, A. K. Akt, FoxO and regulation of apoptosis. *Biochimica et biophysica acta* **1813**, 1978-1986, doi:10.1016/j.bbamcr.2011.03.010 (2011).

127   Faridi, J., Fawcett, J., Wang, L. & Roth, R. A. Akt promotes increased mammalian cell size by stimulating protein synthesis and inhibiting protein degradation. *American journal of physiology. Endocrinology and metabolism* **285**, E964-972, doi:10.1152/ajpendo.00239.2003 (2003).

128   Huang, J. & Manning, B. D. The TSC1-TSC2 complex: a molecular switchboard controlling cell growth. *The Biochemical journal* **412**, 179-190, doi:10.1042/bj20080281 (2008).

129   Skurk, C. *et al.* The FOXO3a transcription factor regulates cardiac myocyte size downstream of AKT signaling. *The Journal of biological chemistry* **280**, 20814-20823, doi:10.1074/jbc.M500528200 (2005).

130   Condorelli, G. *et al.* Akt induces enhanced myocardial contractility and cell size in vivo in transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 12333-12338, doi:10.1073/pnas.172376399 (2002).

131   Skeen, J. E. *et al.* Akt deficiency impairs normal cell proliferation and suppresses oncogenesis in a p53-independent and mTORC1-dependent manner. *Cancer cell* **10**, 269-280, doi:10.1016/j.ccr.2006.08.022 (2006).

132   Zhu, J., Blenis, J. & Yuan, J. Activation of PI3K/Akt and MAPK pathways regulates Myc-mediated transcription by phosphorylating and promoting the degradation of Mad1.

Proceedings of the National Academy of Sciences of the United States of America **105**, 6584-6589, doi:10.1073/pnas.0802785105 (2008).

133   Bouchard, C., Marquardt, J., Bras, A., Medema, R. H. & Eilers, M. Myc-induced proliferation and transformation require Akt-mediated phosphorylation of FoxO proteins. *The EMBO journal* **23**, 2830-2840, doi:10.1038/sj.emboj.7600279 (2004).

134   Mayo, L. D. & Donner, D. B. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 11598-11603, doi:10.1073/pnas.181181198 (2001).

135   Ogawara, Y. *et al.* Akt enhances Mdm2-mediated ubiquitination and degradation of p53. *The Journal of biological chemistry* **277**, 21843-21850, doi:10.1074/jbc.M109745200 (2002).

136   Zhou, B. P. *et al.* Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nature cell biology* **3**, 245-252, doi:10.1038/35060032 (2001).

137   Shin, I. *et al.* PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. *Nature medicine* **8**, 1145-1152, doi:10.1038/nm759 (2002).

138   Cong, L. N. *et al.* Physiological role of Akt in insulin-stimulated translocation of GLUT4 in transfected rat adipose cells. *Molecular endocrinology (Baltimore, Md.)* **11**, 1881-1890, doi:10.1210/mend.11.13.0027 (1997).

139   Sano, H. *et al.* Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *The Journal of biological chemistry* **278**, 14599-14602, doi:10.1074/jbc.C300063200 (2003).

140   Wieman, H. L., Wofford, J. A. & Rathmell, J. C. Cytokine stimulation promotes glucose uptake via phosphatidylinositol-3 kinase/Akt regulation of Glut1 activity and trafficking. *Molecular biology of the cell* **18**, 1437-1446, doi:10.1091/mbc.e06-07-0593 (2007).

141   Embi, N., Rylatt, D. B. & Cohen, P. Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *European journal of biochemistry* **107**, 519-527 (1980).

142   Yecies, J. L. *et al.* Akt stimulates hepatic SREBP1c and lipogenesis through parallel mTORC1-dependent and independent pathways. *Cell metabolism* **14**, 21-32, doi:10.1016/j.cmet.2011.06.002 (2011).

143   Martelli, A. M. *et al.* The emerging multiple roles of nuclear Akt. *Biochimica et biophysica acta* **1823**, 2168-2178, doi:10.1016/j.bbamcr.2012.08.017 (2012).

144   Wang, R. & Brattain, M. G. AKT can be activated in the nucleus. *Cellular signalling* **18**, 1722-1731, doi:10.1016/j.cellsig.2006.01.020 (2006).

145   Jain, M. V. *et al.* Nuclear localized Akt enhances breast cancer stem-like cells through counter-regulation of p21(Waf1/Cip1) and p27(kip1). *Cell cycle (Georgetown, Tex.)* **14**, 2109-2120, doi:10.1080/15384101.2015.1041692 (2015).

146   Ahn, J. Y. *et al.* Nuclear Akt associates with PKC-phosphorylated Ebp1, preventing DNA fragmentation by inhibition of caspase-activated DNase. *The EMBO journal* **25**, 2083-2095, doi:10.1038/sj.emboj.7601111 (2006).

147   Lee, S. B. *et al.* Nuclear Akt interacts with B23/NPM and protects it from proteolytic cleavage, enhancing cell survival. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 16584-16589, doi:10.1073/pnas.0807668105 (2008).

148   Kobayashi, T. & Cohen, P. Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositide 3-kinase is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2. *The Biochemical journal* **339 (Pt 2)**, 319-328 (1999).

149   Aoyama, T. *et al.* Serum and glucocorticoid-responsive kinase-1 regulates cardiomyocyte survival and hypertrophic response. *Circulation* **111**, 1652-1659, doi:10.1161/01.cir.0000160352.58142.06 (2005).

150   Das, S. *et al.* Pathological role of serum- and glucocorticoid-regulated kinase 1 in adverse ventricular remodeling. *Circulation* **126**, 2208-2219, doi:10.1161/circulationaha.112.115592 (2012).

151   Hanna, S. & El-Sibai, M. Signaling networks of Rho GTPases in cell motility. *Cellular signalling* **25**, 1955-1961, doi:10.1016/j.cellsig.2013.04.009 (2013).

152   Patel, V. B. *et al.* PI3Kalpha-regulated gelsolin activity is a critical determinant of cardiac cytoskeletal remodeling and heart disease. *Nature communications* **9**, 5390, doi:10.1038/s41467-018-07812-8 (2018).

153   Fruman, D. A. *et al.* The PI3K Pathway in Human Disease. *Cell* **170**, 605-635, doi:10.1016/j.cell.2017.07.029 (2017).

154   Mora, A. *et al.* Deficiency of PDK1 in cardiac muscle results in heart failure and increased sensitivity to hypoxia. *The EMBO journal* **22**, 4666-4676, doi:10.1093/emboj/cdg469 (2003).

155   Luo, J. *et al.* Class IA phosphoinositide 3-kinase regulates heart size and physiological cardiac hypertrophy. *Molecular and cellular biology* **25**, 9491-9502, doi:10.1128/MCB.25.21.9491-9502.2005 (2005).

156   Crackower, M. A. *et al.* Regulation of myocardial contractility and cell size by distinct PI3K-PTEN signaling pathways. *Cell* **110**, 737-749 (2002).

157   Aikawa, R. *et al.* Insulin prevents cardiomyocytes from oxidative stress-induced apoptosis through activation of PI3 kinase/Akt. *Circulation* **102**, 2873-2879, doi:10.1161/01.cir.102.23.2873 (2000).

158   Maulik, A., Davidson, S. M., Piotrowska, I., Walker, M. & Yellon, D. M. Ischaemic Preconditioning Protects Cardiomyocytes from Anthracycline-Induced Toxicity via the PI3K Pathway. *Cardiovascular drugs and therapy* **32**, 245-253, doi:10.1007/s10557-018-6793-y (2018).

159   Ceci, M. *et al.* Cardiac-specific overexpression of E40K active Akt prevents pressure overload-induced heart failure in mice by increasing angiogenesis and reducing apoptosis. *Cell death and differentiation* **14**, 1060-1062, doi:10.1038/sj.cdd.4402095 (2007).

160   Graves, B. M., Simerly, T., Li, C., Williams, D. L. & Wondergem, R. Phosphoinositide-3-kinase/akt - dependent signaling is required for maintenance of [Ca(2+)](i), I(Ca), and Ca(2+) transients in HL-1 cardiomyocytes. *Journal of biomedical science* **19**, 59, doi:10.1186/1423-0127-19-59 (2012).

161   Catalucci, D. *et al.* Akt regulates L-type Ca2+ channel activity by modulating Cavalpha1 protein stability. *The Journal of cell biology* **184**, 923-933, doi:10.1083/jcb.200805063 (2009).

162   Matsui, T. *et al.* Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia in vivo. *Circulation* **104**, 330-335 (2001).

163   Su, F. *et al.* Cardioprotection by PI3K-mediated signaling is required for anti-arrhythmia and myocardial repair in response to ischemic preconditioning in infarcted pig hearts. *Laboratory investigation; a journal of technical methods and pathology* **95**, 860-871, doi:10.1038/labinvest.2015.64 (2015).

164   Zhu, M. *et al.* Ischemic postconditioning protects remodeled myocardium via the PI3K-PKB/Akt reperfusion injury salvage kinase pathway. *Cardiovascular research* **72**, 152-162, doi:10.1016/j.cardiores.2006.06.027 (2006).

165   Woulfe, K. C. *et al.* Glycogen synthase kinase-3beta regulates post-myocardial infarction remodeling and stress-induced cardiomyocyte proliferation in vivo. *Circulation research* **106**, 1635-1645, doi:10.1161/circresaha.109.211482 (2010).

166   Ahmad, F. *et al.* Cardiomyocyte-specific deletion of Gsk3alpha mitigates post-myocardial infarction remodeling, contractile dysfunction, and heart failure. *Journal of the American College of Cardiology* **64**, 696-706, doi:10.1016/j.jacc.2014.04.068 (2014).

167   Lal, H. *et al.* Cardiac fibroblast glycogen synthase kinase-3beta regulates ventricular remodeling and dysfunction in ischemic heart. *Circulation* **130**, 419-430, doi:10.1161/circulationaha.113.008364 (2014).

168   Talman, V. & Kivela, R. Cardiomyocyte-Endothelial Cell Interactions in Cardiac Remodeling and Regeneration. *Frontiers in cardiovascular medicine* **5**, 101, doi:10.3389/fcvm.2018.00101 (2018).

169   Skurk, C. *et al.* The Akt-regulated forkhead transcription factor FOXO3a controls endothelial cell viability through modulation of the caspase-8 inhibitor FLIP. *The Journal of biological chemistry* **279**, 1513-1525, doi:10.1074/jbc.M304736200 (2004).

170   Kawasaki, K. *et al.* Activation of the phosphatidylinositol 3-kinase/protein kinase Akt pathway mediates nitric oxide-induced endothelial cell migration and angiogenesis. *Molecular and cellular biology* **23**, 5726-5737, doi:10.1128/mcb.23.16.5726-5737.2003 (2003).

171   Ackah, E. *et al.* Akt1/protein kinase Balpha is critical for ischemic and VEGF-mediated angiogenesis. *The Journal of clinical investigation* **115**, 2119-2127, doi:10.1172/jci24726 (2005).

172   Tsuji-Tamura, K. & Ogawa, M. Inhibition of the PI3K-Akt and mTORC1 signaling pathways promotes the elongation of vascular endothelial cells. *Journal of cell science* **129**, 1165-1178, doi:10.1242/jcs.178434 (2016).

173   Tao, Z. *et al.* Coexpression of VEGF and angiopoietin-1 promotes angiogenesis and cardiomyocyte proliferation reduces apoptosis in porcine myocardial infarction (MI) heart. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 2064-2069, doi:10.1073/pnas.1018925108 (2011).

174   Scherrer-Crosbie, M. *et al.* Endothelial nitric oxide synthase limits left ventricular remodeling after myocardial infarction in mice. *Circulation* **104**, 1286-1291, doi:10.1161/hc3601.094298 (2001).

175   Jones, S. P. *et al.* Endothelial nitric oxide synthase overexpression attenuates congestive heart failure in mice. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 4891-4896, doi:10.1073/pnas.0837428100 (2003).

176   Bekhite, M. M. *et al.* VEGF-mediated PI3K class IA and PKC signaling in cardiomyogenesis and vasculogenesis of mouse embryonic stem cells. *Journal of cell science* **124**, 1819-1830, doi:10.1242/jcs.077594 (2011).

177   Naito, A. T. *et al.* Phosphatidylinositol 3-kinase-Akt pathway plays a critical role in early cardiomyogenesis by regulating canonical Wnt signaling. *Circulation research* **97**, 144-151, doi:10.1161/01.RES.0000175241.92285.f8 (2005).

178   Lu, Q. *et al.* Rapamycin efficiently promotes cardiac differentiation of mouse embryonic stem cells. *Bioscience reports* **37**, doi:10.1042/bsr20160552 (2017).

179   Shioi, T. *et al.* The conserved phosphoinositide 3-kinase pathway determines heart size in mice. *The EMBO journal* **19**, 2537-2548 (2000).

180   Lu, Z. *et al.* Loss of cardiac phosphoinositide 3-kinase p110 alpha results in contractile dysfunction. *Circulation* **120**, 318-325, doi:10.1161/circulationaha.109.873380 (2009).

181   Yano, N. *et al.* Temporally controlled overexpression of cardiac-specific PI3Kalpha induces enhanced myocardial contractility--a new transgenic model. *American journal of physiology. Heart and circulatory physiology* **295**, H1690-1694, doi:10.1152/ajpheart.00531.2008 (2008).

182   Waardenberg, A. J. *et al.* Phosphoinositide 3-kinase (PI3K(p110alpha)) directly regulates key components of the Z-disc and cardiac structure. *The Journal of biological chemistry* **286**, 30837-30846, doi:10.1074/jbc.M111.271684 (2011).

183   Braz, J. C. *et al.* Selective activation of PI3Kalpha/Akt/GSK-3beta signalling and cardiac compensatory hypertrophy during recovery from heart failure. *European journal of heart failure* **11**, 739-748, doi:10.1093/eurjhf/hfp094 (2009).

184   McMullen, J. R. *et al.* Protective effects of exercise and phosphoinositide 3-kinase(p110alpha) signaling in dilated and hypertrophic cardiomyopathy. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 612-617, doi:10.1073/pnas.0606663104 (2007).

185   Weeks, K. L. *et al.* Phosphoinositide 3-kinase p110alpha is a master regulator of exercise-induced cardioprotection and PI3K gene therapy rescues cardiac dysfunction. *Circulation. Heart failure* **5**, 523-534, doi:10.1161/circheartfailure.112.966622 (2012).

186   Pretorius, L. *et al.* Reduced phosphoinositide 3-kinase (p110alpha) activation increases the susceptibility to atrial fibrillation. *The American journal of pathology* **175**, 998-1009, doi:10.2353/ajpath.2009.090126 (2009).

187   Prakoso, D. *et al.* Phosphoinositide 3-kinase (p110alpha) gene delivery limits diabetes-induced cardiac NADPH oxidase and cardiomyopathy in a mouse model with established diastolic dysfunction. *Clinical science (London, England : 1979)* **131**, 1345-1360, doi:10.1042/cs20170063 (2017).

188   Lin, R. C. *et al.* PI3K(p110 alpha) protects against myocardial infarction-induced heart failure: identification of PI3K-regulated miRNA and mRNA. *Arteriosclerosis, thrombosis, and vascular biology* **30**, 724-732, doi:10.1161/atvbaha.109.201988 (2010).

189   Graupera, M. *et al.* Angiogenesis selectively requires the p110alpha isoform of PI3K to control endothelial cell migration. *Nature* **453**, 662-666, doi:10.1038/nature06892 (2008).

190   Dimmeler, S., Dernbach, E. & Zeiher, A. M. Phosphorylation of the endothelial nitric oxide synthase at ser-1177 is required for VEGF-induced endothelial cell migration. *FEBS letters* **477**, 258-262 (2000).

191   Abid, M. R. *et al.* Vascular endothelial growth factor activates PI3K/Akt/forkhead signaling in endothelial cells. *Arteriosclerosis, thrombosis, and vascular biology* **24**, 294-300, doi:10.1161/01.atv.0000110502.10593.06 (2004).

192   Cain, R. J., Vanhaesebroeck, B. & Ridley, A. J. The PI3K p110alpha isoform regulates endothelial adherens junctions via Pyk2 and Rac1. *The Journal of cell biology* **188**, 863-876, doi:10.1083/jcb.200907135 (2010).

193   Cain, R. J., Vanhaesebroeck, B. & Ridley, A. J. Different PI 3-kinase inhibitors have distinct effects on endothelial permeability and leukocyte transmigration. *The international journal of biochemistry & cell biology* **44**, 1929-1936, doi:10.1016/j.biocel.2012.07.009 (2012).

194   Rossello, X. *et al.* The role of PI3Kalpha isoform in cardioprotection. *Basic research in cardiology* **112**, 66, doi:10.1007/s00395-017-0657-7 (2017).

195   Heller, R. *et al.* Overlapping and distinct roles for PI3Kbeta and gamma isoforms in S1P-induced migration of human and mouse endothelial cells. *Cardiovascular research* **80**, 96-105, doi:10.1093/cvr/cvn159 (2008).

196   Theilmeier, G. *et al.* High-density lipoproteins and their constituent, sphingosine-1-phosphate, directly protect the heart against ischemia/reperfusion injury in vivo via the S1P3 lysophospholipid receptor. *Circulation* **114**, 1403-1409, doi:10.1161/circulationaha.105.607135 (2006).

197   Lin, Z. *et al.* Pi3kcb links Hippo-YAP and PI3K-AKT signaling pathways to promote cardiomyocyte proliferation and survival. *Circulation research* **116**, 35-45, doi:10.1161/CIRCRESAHA.115.304457 (2015).

198   Vadas, O. *et al.* Molecular determinants of PI3Kgamma-mediated activation downstream of G-protein-coupled receptors (GPCRs). *Proceedings of the National Academy of Sciences of the United States of America* **110**, 18862-18867, doi:10.1073/pnas.1304801110 (2013).

199   Naga Prasad, S. V., Barak, L. S., Rapacciuolo, A., Caron, M. G. & Rockman, H. A. Agonist-dependent recruitment of phosphoinositide 3-kinase to the membrane by beta-adrenergic receptor kinase 1. A role in receptor sequestration. *The Journal of biological chemistry* **276**, 18953-18959, doi:10.1074/jbc.M102376200 (2001).

200   Siragusa, M. *et al.* Involvement of phosphoinositide 3-kinase gamma in angiogenesis and healing of experimental myocardial infarction in mice. *Circulation research* **106**, 757-768, doi:10.1161/circresaha.109.207449 (2010).

201   Huang, X. *et al.* Endothelial p110gammaPI3K Mediates Endothelial Regeneration and Vascular Repair After Inflammatory Vascular Injury. *Circulation* **133**, 1093-1103, doi:10.1161/circulationaha.115.020918 (2016).

202   LoRusso, P. M. Inhibition of the PI3K/AKT/mTOR Pathway in Solid Tumors. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **34**, 3803-3815, doi:10.1200/jco.2014.59.0018 (2016).

203   Hopkins, B. D. *et al.* Suppression of insulin feedback enhances the efficacy of PI3K inhibitors. *Nature* **560**, 499-503, doi:10.1038/s41586-018-0343-4 (2018).

204   Fritsch, C. *et al.* Characterization of the novel and specific PI3Kalpha inhibitor NVP-BYL719 and development of the patient stratification strategy for clinical trials. *Molecular cancer therapeutics* **13**, 1117-1129, doi:10.1158/1535-7163.mct-13-0865 (2014).

205   Mayer, I. A. *et al.* A Phase Ib Study of Alpelisib (BYL719), a PI3Kalpha-Specific Inhibitor, with Letrozole in ER+/HER2- Metastatic Breast Cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **23**, 26-34, doi:10.1158/1078-0432.ccr-16-0134 (2017).

206   Juric, D. *et al.* Phosphatidylinositol 3-Kinase alpha-Selective Inhibition With Alpelisib (BYL719) in PIK3CA-Altered Solid Tumors: Results From the First-in-Human Study. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **36**, 1291-1299, doi:10.1200/jco.2017.72.7107 (2018).

207   Andre, F. *et al.* Alpelisib for PIK3CA-Mutated, Hormone Receptor-Positive Advanced Breast Cancer. *The New England journal of medicine* **380**, 1929-1940, doi:10.1056/NEJMoa1813904 (2019).

208   Ni, J. *et al.* Functional characterization of an isoform-selective inhibitor of PI3K-p110beta as a potential anticancer agent. *Cancer discovery* **2**, 425-433, doi:10.1158/2159-8290.cd-12-0003 (2012).

209   Feng, C. *et al.* PI3Kbeta inhibitor TGX221 selectively inhibits renal cell carcinoma cells with both VHL and SETD2 mutations and links multiple pathways. *Scientific reports* **5**, 9465, doi:10.1038/srep09465 (2015).

210   Mateo, J. *et al.* A First-Time-in-Human Study of GSK2636771, a Phosphoinositide 3 Kinase Beta-Selective Inhibitor, in Patients with Advanced Solid Tumors. *Clinical cancer research : an official journal of the American Association for Cancer Research* **23**, 5981-5992, doi:10.1158/1078-0432.ccr-17-0725 (2017).

211   Ballou, L. M., Lin, R. Z. & Cohen, I. S. Control of cardiac repolarization by phosphoinositide 3-kinase signaling to ion channels. *Circulation research* **116**, 127-137, doi:10.1161/CIRCRESAHA.116.303975 (2015).

212   Zhabyeyev, P., McLean, B., Chen, X., Vanhaesebroeck, B. & Oudit, G. Y. Inhibition of PI3Kinase-alpha is pro-arrhythmic and associated with enhanced late Na(+) current, contractility, and Ca(2+) release in murine hearts. *Journal of molecular and cellular cardiology* **132**, 98-109, doi:10.1016/j.yjmcc.2019.05.008 (2019).

213   Jain, S. *et al.* Phase I study of alpelisib (BYL-719) and trastuzumab emtansine (T-DM1) in HER2-positive metastatic breast cancer (MBC) after trastuzumab and taxane therapy. *Breast cancer research and treatment* **171**, 371-381, doi:10.1007/s10549-018-4792-0 (2018).

214   McLean, B. A. *et al.* PI3Kalpha is essential for the recovery from Cre/tamoxifen cardiotoxicity and in myocardial insulin signalling but is not required for normal myocardial contractility in the adult heart. *Cardiovascular research* **105**, 292-303, doi:10.1093/cvr/cvv016 (2015).

215   McLean, B. A. *et al.* PI3Kalpha Pathway Inhibition With Doxorubicin Treatment Results in Distinct Biventricular Atrophy and Remodeling With Right Ventricular Dysfunction. *Journal of the American Heart Association* **8**, e010961, doi:10.1161/jaha.118.010961 (2019).

216   Haddad, G. *et al.* Phosphoinositide 3-kinase beta mediates microvascular endothelial repair of thrombotic microangiopathy. *Blood* **124**, 2142-2149, doi:10.1182/blood-2014-02-557975 (2014).

217   Kulkarni, S. *et al.* PI3Kbeta plays a critical role in neutrophil activation by immune complexes. *Science signaling* **4**, ra23, doi:10.1126/scisignal.2001617 (2011).

218   Wang, W. *et al.* Loss of Apelin exacerbates myocardial infarction adverse remodeling and ischemia-reperfusion injury: therapeutic potential of synthetic Apelin analogues. *Journal of the American Heart Association* **2**, e000249, doi:10.1161/JAHA.113.000249 (2013).

219   Xu, Z., Alloush, J., Beck, E. & Weisleder, N. A murine model of myocardial ischemia-reperfusion injury through ligation of the left anterior descending artery. *Journal of visualized experiments : JoVE* **86**, 51329, doi:10.3791/51329 (2014).

220   Cerqueira, M. *et al.* Standardized myocardial segmentation and nomenclature for tomographic imaging of the heart: A statement for healthcare professionals from the Cardiac Imaging Committee of the Council on Clinical Cardiology of the American Heart Association. *Circulation* **105**, 539-542, doi:10.1067/mnc.2002.123122 (2002).

221   Bauer, M. *et al.* Echocardiographic Speckle-Tracking Based Strain Imaging for Rapid Cardiovascular Phenotyping in Mice. *Circulation research* **108**, 908-916, doi:10.1161/CIRCRESAHA.110.239574 (2011).

222   Zhang, Q. X. *et al.* Glomerular endothelial PI3 kinase-alpha couples to VEGFR2, but is not required for eNOS activation. *American journal of physiology. Renal physiology* **301**, F1242-1250, doi:10.1152/ajprenal.00662.2010 (2011).

223   Wagner, M. *et al.* Isolation and intravenous injection of murine bone marrow derived monocytes. *Journal of visualized experiments : JoVE*, doi:10.3791/52347 (2014).

224   Li, D., Wu, J., Bai, Y., Zhao, X. & Liu, L. Isolation and culture of adult mouse cardiomyocytes for cell signaling and in vitro cardiac hypertrophy. *Journal of visualized experiments : JoVE*, doi:10.3791/51357 (2014).

225   Patel, V. B. *et al.* Loss of p47phox subunit enhances susceptibility to biomechanical stress and heart failure because of dysregulation of cortactin and actin filaments. *Circulation research* **112**, 1542-1556, doi:10.1161/circresaha.111.300299 (2013).

226   Dimauro, I., Pearson, T., Caporossi, D. & Jackson, M. J. A simple protocol for the subcellular fractionation of skeletal muscle cells and tissue. *BMC research notes* **5**, 513, doi:10.1186/1756-0500-5-513 (2012).

227   Hall, D. D. *et al.* Ectopic expression of Cdk8 induces eccentric hypertrophy and heart failure. *JCI Insight* **2**, doi:10.1172/jci.insight.92476 (2017).

228   Liu, P., Cheng, H., Roberts, T. M. & Zhao, J. J. Targeting the phosphoinositide 3-kinase pathway in cancer. *Nature reviews. Drug discovery* **8**, 627-644, doi:10.1038/nrd2926 (2009).

229   Huang, X., Liu, G., Guo, J. & Su, Z. The PI3K/AKT pathway in obesity and type 2 diabetes. *International journal of biological sciences* **14**, 1483-1496, doi:10.7150/ijbs.27173 (2018).

230   Madsen, R. R., Vanhaesebroeck, B. & Semple, R. K. Cancer-Associated PIK3CA Mutations in Overgrowth Disorders. *Trends in molecular medicine* **24**, 856-870, doi:10.1016/j.molmed.2018.08.003 (2018).

231   Mizrachi, A. *et al.* Tumour-specific PI3K inhibition via nanoparticle-targeted delivery in head and neck squamous cell carcinoma. *Nature communications* **8**, 14292, doi:10.1038/ncomms14292 (2017).

232   James, A. *et al.* Absorption, distribution, metabolism, and excretion of [(14)C]BYL719 (alpelisib) in healthy male volunteers. *Cancer chemotherapy and pharmacology* **76**, 751-760, doi:10.1007/s00280-015-2842-4 (2015).

233   Venot, Q. *et al.* Targeted therapy in patients with PIK3CA-related overgrowth syndrome. *Nature* **558**, 540-546, doi:10.1038/s41586-018-0217-9 (2018).

234   Lopez-Guadamillas, E. *et al.* PI3Kalpha inhibition reduces obesity in mice. *Aging* **8**, 2747-2753, doi:10.18632/aging.101075 (2016).

235   Song, N. J. *et al.* PI3Ka-Akt1-mediated Prdm4 induction in adipose tissue increases energy expenditure, inhibits weight gain, and improves insulin resistance in diet-induced obese mice. *Cell death & disease* **9**, 876, doi:10.1038/s41419-018-0904-3 (2018).

236   Holy, E. W. *et al.* PI3K/p110alpha inhibition selectively interferes with arterial thrombosis and neointima formation, but not re-endothelialization: potential implications for drug-eluting stent design. *European heart journal* **35**, 808-820, doi:10.1093/eurheartj/eht496 (2014).

237   Prabhu, S. D. & Frangogiannis, N. G. The Biological Basis for Cardiac Repair After Myocardial Infarction: From Inflammation to Fibrosis. *Circulation research* **119**, 91-112, doi:10.1161/circresaha.116.303577 (2016).

238   Crackower, M. A. *et al.* Regulation of Myocardial Contractility and Cell Size by Distinct PI3K-PTEN Signaling Pathways. *Cell* **110**, 737-749 (2002).

239   Chen, X. *et al.* Endothelial and cardiomyocyte PI3Kbeta divergently regulate cardiac remodelling in response to ischaemic injury. *Cardiovascular research* **115**, 1343-1356, doi:10.1093/cvr/cvy298 (2019).

240   Gobin, B. *et al.* BYL719, a new alpha-specific PI3K inhibitor: single administration and in combination with conventional chemotherapy for the treatment of osteosarcoma. *International journal of cancer* **136**, 784-796, doi:10.1002/ijc.29040 (2015).

241   Elkabets, M. *et al.* AXL mediates resistance to PI3Kalpha inhibition by activating the EGFR/PKC/mTOR axis in head and neck and esophageal squamous cell carcinomas. *Cancer cell* **27**, 533-546, doi:10.1016/j.ccell.2015.03.010 (2015).

242   Duarte, C. W., Lindner, V., Francis, S. A. & Schoormans, D. Visualization of Cancer and Cardiovascular Disease Co-Occurrence With Network Methods. *JCO clinical cancer informatics* **1**, 1-12, doi:10.1200/cci.16.00071 (2017).

243   Vincent, L., Leedy, D., Masri, S. C. & Cheng, R. K. Cardiovascular Disease and Cancer: Is There Increasing Overlap? *Current oncology reports* **21**, 47, doi:10.1007/s11912-019-0796-0 (2019).

244   Zoller, B., Ji, J., Sundquist, J. & Sundquist, K. Risk of coronary heart disease in patients with cancer: a nationwide follow-up study from Sweden. *European journal of cancer (Oxford, England : 1990)* **48**, 121-128, doi:10.1016/j.ejca.2011.09.015 (2012).

245   Hasin, T. *et al.* Heart Failure After Myocardial Infarction Is Associated With Increased Risk of Cancer. *Journal of the American College of Cardiology* **68**, 265-271, doi:10.1016/j.jacc.2016.04.053 (2016).

246   Koene, R. J., Prizment, A. E., Blaes, A. & Konety, S. H. Shared Risk Factors in Cardiovascular Disease and Cancer. *Circulation* **133**, 1104-1114, doi:10.1161/circulationaha.115.020406 (2016).

247   Johnson, C. B., Davis, M. K., Law, A. & Sulpher, J. Shared Risk Factors for Cardiovascular Disease and Cancer: Implications for Preventive Health and Clinical Care in Oncology Patients. *The Canadian journal of cardiology* **32**, 900-907, doi:10.1016/j.cjca.2016.04.008 (2016).

248   Meijers, W. C. *et al.* Heart Failure Stimulates Tumor Growth by Circulating Factors. *Circulation* **138**, 678-691, doi:10.1161/circulationaha.117.030816 (2018).

249   Mayer, I. A. *et al.* A Phase II Randomized Study of Neoadjuvant Letrozole Plus Alpelisib for Hormone Receptor-Positive, Human Epidermal Growth Factor Receptor 2-Negative Breast Cancer (NEO-ORB). *Clinical cancer research : an official journal of the American*

Association for Cancer Research **25**, 2975-2987, doi:10.1158/1078-0432.ccr-18-3160 (2019).

250 Juric, D. *et al.* Alpelisib Plus Fulvestrant in PIK3CA-Altered and PIK3CA-Wild-Type Estrogen Receptor-Positive Advanced Breast Cancer: A Phase 1b Clinical Trial. *JAMA oncology*, e184475, doi:10.1001/jamaoncol.2018.4475 (2018).

251 Juric, D. *et al.* A First-in-Human, Phase I, Dose-Escalation Study of TAK-117, a Selective PI3Kalpha Isoform Inhibitor, in Patients with Advanced Solid Malignancies. *Clinical cancer research : an official journal of the American Association for Cancer Research* **23**, 5015-5023, doi:10.1158/1078-0432.ccr-16-2888 (2017).

252 Srikanthan, P., Horwich, T. B. & Tseng, C. H. Relation of Muscle Mass and Fat Mass to Cardiovascular Disease Mortality. *The American journal of cardiology* **117**, 1355-1360, doi:10.1016/j.amjcard.2016.01.033 (2016).

253 Esposito, A., Viale, G. & Curigliano, G. Safety, Tolerability, and Management of Toxic Effects of Phosphatidylinositol 3-Kinase Inhibitor Treatment in Patients With Cancer: A Review. *JAMA oncology*, doi:10.1001/jamaoncol.2019.0034 (2019).

254 Nielsen, J. B. *et al.* Risk prediction of cardiovascular death based on the QTc interval: evaluating age and gender differences in a large primary care population. *European heart journal* **35**, 1335-1344, doi:10.1093/eurheartj/ehu081 (2014).

255 Biering-Sorensen, T. *et al.* Global Longitudinal Strain by Echocardiography Predicts Long-Term Risk of Cardiovascular Morbidity and Mortality in a Low-Risk General Population: The Copenhagen City Heart Study. *Circulation. Cardiovascular imaging* **10**, doi:10.1161/circimaging.116.005521 (2017).

256 Chugh, S. S. *et al.* Determinants of prolonged QT interval and their contribution to sudden death risk in coronary artery disease: the Oregon Sudden Unexpected Death Study. *Circulation* **119**, 663-670, doi:10.1161/circulationaha.108.797035 (2009).

257 Burchfield, J. S., Xie, M. & Hill, J. A. Pathological ventricular remodeling: mechanisms: part 1 of 2. *Circulation* **128**, 388-400, doi:10.1161/circulationaha.113.001878 (2013).

258 Ahn, A., Frishman, W. H., Gutwein, A., Passeri, J. & Nelson, M. Therapeutic angiogenesis: a new treatment approach for ischemic heart disease--part I. *Cardiology in review* **16**, 163-171, doi:10.1097/CRD.0b013e3181620e3b (2008).

259 Xu, R., Sun, Y., Chen, Z., Yao, Y. & Ma, G. Hypoxic Preconditioning Inhibits Hypoxia-induced Apoptosis of Cardiac Progenitor Cells via the PI3K/Akt-DNMT1-p53 Pathway. *Scientific reports* **6**, 30922, doi:10.1038/srep30922 (2016).

260   Chang, H. *et al.* QSKL protects against myocardial apoptosis on heart failure via PI3K/Akt-p53 signaling pathway. *Scientific reports* **7**, 16986, doi:10.1038/s41598-017-17163-x (2017).

261   Zhu, W. *et al.* MicroRNA-503 regulates hypoxia-induced cardiomyocytes apoptosis through PI3K/Akt pathway by targeting IGF-1R. *Biochemical and biophysical research communications* **506**, 1026-1031, doi:10.1016/j.bbrc.2018.10.160 (2018).

262   Fujio, Y. & Walsh, K. Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. *The Journal of biological chemistry* **274**, 16349-16354, doi:10.1074/jbc.274.23.16349 (1999).

263   Lee, M. Y. *et al.* Endothelial Akt1 mediates angiogenesis by phosphorylating multiple angiogenic substrates. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 12865-12870, doi:10.1073/pnas.1408472111 (2014).

264   Manoury, B., Montiel, V. & Balligand, J. L. Nitric oxide synthase in post-ischaemic remodelling: new pathways and mechanisms. *Cardiovascular research* **94**, 304-315, doi:10.1093/cvr/cvr360 (2012).

265   Matsui, T. *et al.* Adenoviral gene transfer of activated phosphatidylinositol 3'-kinase and Akt inhibits apoptosis of hypoxic cardiomyocytes in vitro. *Circulation* **100**, 2373-2379, doi:10.1161/01.cir.100.23.2373 (1999).

266   Mehrhof, F. B. *et al.* In cardiomyocyte hypoxia, insulin-like growth factor-I-induced antiapoptotic signaling requires phosphatidylinositol-3-OH-kinase-dependent and mitogen-activated protein kinase-dependent activation of the transcription factor cAMP response element-binding protein. *Circulation* **104**, 2088-2094, doi:10.1161/hc4201.097133 (2001).

267   Song, H. P., Chu, Z. G., Zhang, D. X., Dang, Y. M. & Zhang, Q. PI3K-AKT Pathway Protects Cardiomyocytes Against Hypoxia-Induced Apoptosis by MitoKATP-Mediated Mitochondrial Translocation of pAKT. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* **49**, 717-727, doi:10.1159/000493037 (2018).

268   Matsui, T. & Rosenzweig, A. Convergent signal transduction pathways controlling cardiomyocyte survival and function: the role of PI 3-kinase and Akt. *Journal of molecular and cellular cardiology* **38**, 63-71, doi:10.1016/j.yjmcc.2004.11.005 (2005).

269   Roth, G. A. *et al.* Estimates of global and regional premature cardiovascular mortality in 2025. *Circulation* **132**, 1270-1282, doi:10.1161/circulationaha.115.016021 (2015).

270   St John Sutton, M. G. & Sharpe, N. Left Ventricular Remodeling After Myocardial Infarction - Pathophysiology and Therapy. *Circulation* **101**, 2981-2988 (2000).

271   Yellon, D. M. & Hausenloy, D. J. Myocardial reperfusion injury. *The New England journal of medicine* **357**, 1121-1135, doi:10.1056/NEJMra071667 (2007).

272   Ferrara, N. & Kerbel, R. S. Angiogenesis as a therapeutic target. *Nature* **438**, 967-974, doi:10.1038/nature04483 (2005).

273   Datta, S. R., Brunet, A. & Greenberg, M. E. Cellular survival: a play in three Akts. *Genes & development* **13**, 2905-2927 (1999).

274   Hall, D. D. *et al.* Ectopic expression of Cdk8 induces eccentric hypertrophy and heart failure. *JCI insight* **2**, e92476, doi:10.1172/jci.insight.92476 (2017).

275   Sussman, M. A. *et al.* Myocardial AKT: the omnipresent nexus. *Physiological reviews* **91**, 1023-1070, doi:10.1152/physrev.00024.2010 (2011).

276   Simons, M., Gordon, E. & Claesson-Welsh, L. Mechanisms and regulation of endothelial VEGF receptor signalling. *Nature reviews. Molecular cell biology* **17**, 611-625, doi:10.1038/nrm.2016.87 (2016).

277   Lee, S. *et al.* Autocrine VEGF signaling is required for vascular homeostasis. *Cell* **130**, 691-703, doi:10.1016/j.cell.2007.06.054 (2007).

278   Small, E. M. *et al.* Myocardin-related transcription factor-a controls myofibroblast activation and fibrosis in response to myocardial infarction. *Circulation research* **107**, 294-304, doi:10.1161/circresaha.110.223172 (2010).

279   Fraccarollo, D. *et al.* Deletion of cardiomyocyte mineralocorticoid receptor ameliorates adverse remodeling after myocardial infarction. *Circulation* **123**, 400-408, doi:10.1161/circulationaha.110.983023 (2011).

280   Euler-Taimor, G. & Heger, J. The complex pattern of SMAD signaling in the cardiovascular system. *Cardiovascular research* **69**, 15-25, doi:10.1016/j.cardiores.2005.07.007 (2006).

281   Kunkel, G. H., Chaturvedi, P. & Tyagi, S. C. Mitochondrial pathways to cardiac recovery: TFAM. *Heart failure reviews* **21**, 499-517, doi:10.1007/s10741-016-9561-8 (2016).

282   Finck, B. N. The PPAR regulatory system in cardiac physiology and disease. *Cardiovascular research* **73**, 269-277, doi:10.1016/j.cardiores.2006.08.023 (2007).

283   McConnell, B. B. & Yang, V. W. Mammalian Kruppel-like factors in health and diseases. *Physiological reviews* **90**, 1337-1381, doi:10.1152/physrev.00058.2009 (2010).

284   Hausenloy, D. J. & Yellon, D. M. Myocardial ischemia-reperfusion injury: a neglected therapeutic target. *The Journal of clinical investigation* **123**, 92-100, doi:10.1172/jci62874 (2013).

285   Morello, F., Perino, A. & Hirsch, E. Phosphoinositide 3-kinase signalling in the vascular system. *Cardiovascular research* **82**, 261-271, doi:10.1093/cvr/cvn325 (2009).

286   Costa, C. *et al.* Measurement of PIP3 levels reveals an unexpected role for p110beta in early adaptive responses to p110alpha-specific inhibitors in luminal breast cancer. *Cancer cell* **27**, 97-108, doi:10.1016/j.ccell.2014.11.007 (2015).

287   Schwartz, S. *et al.* Feedback suppression of PI3Kalpha signaling in PTEN-mutated tumors is relieved by selective inhibition of PI3Kbeta. *Cancer cell* **27**, 109-122, doi:10.1016/j.ccell.2014.11.008 (2015).

288   Condorelli, G. *et al.* Heart-targeted overexpression of caspase3 in mice increases infarct size and depresses cardiac function. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 9977-9982, doi:10.1073/pnas.161120198 (2001).

289   Luedde, M. *et al.* RIP3, a kinase promoting necroptotic cell death, mediates adverse remodelling after myocardial infarction. *Cardiovascular research* **103**, 206-216, doi:10.1093/cvr/cvu146 (2014).

290   Shiraishi, I. *et al.* Nuclear targeting of Akt enhances kinase activity and survival of cardiomyocytes. *Circulation research* **94**, 884-891, doi:10.1161/01.RES.0000124394.01180.BE (2004).

291   Wang, J. C., Normand, S. L., Mauri, L. & Kuntz, R. E. Coronary artery spatial distribution of acute myocardial infarction occlusions. *Circulation* **110**, 278-284, doi:10.1161/01.cir.0000135468.67850.f4 (2004).

292   Entezarjou, A., Mohammad, M. A., Andell, P. & Koul, S. Culprit vessel: impact on short-term and long-term prognosis in patients with ST-elevation myocardial infarction. *Open heart* **5**, e000852, doi:10.1136/openhrt-2018-000852 (2018).

293   Patel, C. G. *et al.* Characterizing the Sources of Pharmacokinetic Variability for TAK-117 (Serabelisib), an Investigational Phosphoinositide 3-Kinase Alpha Inhibitor: A Clinical Biopharmaceutics Study to Inform Development Strategy. *Clinical pharmacology in drug development* **8**, 637-646, doi:10.1002/cpdd.613 (2019).

294   Luis, S. A., Chan, J. & Pellikka, P. A. Echocardiographic Assessment of Left Ventricular Systolic Function: An Overview of Contemporary Techniques, Including Speckle-Tracking Echocardiography. *Mayo Clinic proceedings* **94**, 125-138, doi:10.1016/j.mayocp.2018.07.017 (2019).

295   Garcia-Galiano, D. *et al.* PI3Kalpha inactivation in leptin receptor cells increases leptin sensitivity but disrupts growth and reproduction. *JCI insight* **2**, doi:10.1172/jci.insight.96728 (2017).

296   Glass, D. J. PI3 kinase regulation of skeletal muscle hypertrophy and atrophy. *Current topics in microbiology and immunology* **346**, 267-278, doi:10.1007/82_2010_78 (2010).

297   Ortega-Molina, A. *et al.* Pharmacological inhibition of PI3K reduces adiposity and metabolic syndrome in obese mice and rhesus monkeys. *Cell metabolism* **21**, 558-570, doi:10.1016/j.cmet.2015.02.017 (2015).

298   Nelson, V. L., Jiang, Y. P., Dickman, K. G., Ballou, L. M. & Lin, R. Z. Adipose tissue insulin resistance due to loss of PI3K p110alpha leads to decreased energy expenditure and obesity. *American journal of physiology. Endocrinology and metabolism* **306**, E1205-1216, doi:10.1152/ajpendo.00625.2013 (2014).

299   Blair, T. A. *et al.* Phosphoinositide 3-kinases p110alpha and p110beta have differential roles in insulin-like growth factor-1-mediated Akt phosphorylation and platelet priming. *Arteriosclerosis, thrombosis, and vascular biology* **34**, 1681-1688, doi:10.1161/atvbaha.114.303954 (2014).

300   van Geel, R. *et al.* A Phase Ib Dose-Escalation Study of Encorafenib and Cetuximab with or without Alpelisib in Metastatic BRAF-Mutant Colorectal Cancer. *Cancer discovery* **7**, 610-619, doi:10.1158/2159-8290.cd-16-0795 (2017).

301   Konstantinopoulos, P. A. *et al.* Olaparib and alpha-specific PI3K inhibitor alpelisib for patients with epithelial ovarian cancer: a dose-escalation and dose-expansion phase 1b trial. *The Lancet. Oncology* **20**, 570-580, doi:10.1016/s1470-2045(18)30905-7 (2019).

302   Malmborg, M. *et al.* Incidence of new onset cancer in patients with a myocardial infarction - a nationwide cohort study. *BMC cardiovascular disorders* **18**, 198, doi:10.1186/s12872-018-0932-z (2018).

303   Korff, T., Kimmina, S., Martiny-Baron, G. & Augustin, H. G. Blood vessel maturation in a 3-dimensional spheroidal coculture model: direct contact with smooth muscle cells regulates endothelial cell quiescence and abrogates VEGF responsiveness. *Faseb j* **15**, 447-457, doi:10.1096/fj.00-0139com (2001).

304   Lee, S. H. *et al.* Early expression of angiogenesis factors in acute myocardial ischemia and infarction. *The New England journal of medicine* **342**, 626-633, doi:10.1056/nejm200003023420904 (2000).

305   Hojo, Y. *et al.* Expression of vascular endothelial growth factor in patients with acute myocardial infarction. *Journal of the American College of Cardiology* **35**, 968-973, doi:10.1016/s0735-1097(99)00632-4 (2000).

306   Segers, V. F. M., Brutsaert, D. L. & De Keulenaer, G. W. Cardiac Remodeling: Endothelial Cells Have More to Say Than Just NO. *Frontiers in physiology* **9**, 382, doi:10.3389/fphys.2018.00382 (2018).

307   Kerkela, R., Woulfe, K. & Force, T. Glycogen synthase kinase-3beta -- actively inhibiting hypertrophy. *Trends in cardiovascular medicine* **17**, 91-96, doi:10.1016/j.tcm.2007.01.004 (2007).

308   Kitamura, Y. *et al.* Manipulation of cardiac phosphatidylinositol 3-kinase (PI3K)/Akt signaling by apoptosis regulator through modulating IAP expression (ARIA) regulates cardiomyocyte death during doxorubicin-induced cardiomyopathy. *The Journal of biological chemistry* **289**, 2788-2800, doi:10.1074/jbc.M113.508143 (2014).

309   Dhanasekaran, A. *et al.* Multiple antiapoptotic targets of the PI3K/Akt survival pathway are activated by epoxyeicosatrienoic acids to protect cardiomyocytes from hypoxia/anoxia. *American journal of physiology. Heart and circulatory physiology* **294**, H724-735, doi:10.1152/ajpheart.00979.2007 (2008).

310   Sun, J. *et al.* Ginsenoside RK3 Prevents Hypoxia-Reoxygenation Induced Apoptosis in H9c2 Cardiomyocytes via AKT and MAPK Pathway. *Evidence-based complementary and alternative medicine : eCAM* **2013**, 690190, doi:10.1155/2013/690190 (2013).

311   Shimizu, I. & Minamino, T. Physiological and pathological cardiac hypertrophy. *Journal of molecular and cellular cardiology* **97**, 245-262, doi:10.1016/j.yjmcc.2016.06.001 (2016).

312   Liu, C. L. *et al.* Salubrinal protects against tunicamycin and hypoxia induced cardiomyocyte apoptosis via the PERK-eIF2alpha signaling pathway. *Journal of geriatric cardiology : JGC* **9**, 258-268, doi:10.3724/sp.j.1263.2012.02292 (2012).

313   Crea, F. & Libby, P. Acute Coronary Syndromes: The Way Forward From Mechanisms to Precision Treatment. *Circulation* **136**, 1155-1166, doi:10.1161/circulationaha.117.029870 (2017).

314   Badimon, L. & Vilahur, G. Thrombosis formation on atherosclerotic lesions and plaque rupture. *Journal of internal medicine* **276**, 618-632, doi:10.1111/joim.12296 (2014).

315   Dutta, P. *et al.* Myocardial infarction accelerates atherosclerosis. *Nature* **487**, 325-329, doi:10.1038/nature11260 (2012).

316 Eisenreich, A. & Rauch, U. PI3K inhibitors in cardiovascular disease. *Cardiovascular therapeutics* **29**, 29-36, doi:10.1111/j.1755-5922.2010.00206.x (2011).

317 Gilio, K. *et al.* Non-redundant roles of phosphoinositide 3-kinase isoforms alpha and beta in glycoprotein VI-induced platelet signaling and thrombus formation. *The Journal of biological chemistry* **284**, 33750-33762, doi:10.1074/jbc.M109.048439 (2009).

318 Aird, W. C. Endothelial cell heterogeneity. *Cold Spring Harbor perspectives in medicine* **2**, a006429, doi:10.1101/cshperspect.a006429 (2012).

319 Jackson, S. P. *et al.* PI 3-kinase p110beta: a new target for antithrombotic therapy. *Nature medicine* **11**, 507-514, doi:10.1038/nm1232 (2005).

320 Manganaro, D. *et al.* Activation of phosphatidylinositol 3-kinase beta by the platelet collagen receptors integrin alpha2beta1 and GPVI: The role of Pyk2 and c-Cbl. *Biochimica et biophysica acta* **1853**, 1879-1888, doi:10.1016/j.bbamcr.2015.05.004 (2015).

321 Laurent, P. A. *et al.* Platelet PI3Kbeta and GSK3 regulate thrombus stability at a high shear rate. *Blood* **125**, 881-888, doi:10.1182/blood-2014-07-588335 (2015).

322 Nylander, S., Wagberg, F., Andersson, M., Skarby, T. & Gustafsson, D. Exploration of efficacy and bleeding with combined phosphoinositide 3-kinase beta inhibition and aspirin in man. *Journal of thrombosis and haemostasis : JTH* **13**, 1494-1502, doi:10.1111/jth.13027 (2015).

323 Bird, J. E., Smith, P. L., Bostwick, J. S., Shipkova, P. & Schumacher, W. A. Bleeding response induced by anti-thrombotic doses of a phosphoinositide 3-kinase (PI3K)-beta inhibitor in mice. *Thrombosis research* **127**, 560-564, doi:10.1016/j.thromres.2011.02.007 (2011).

324 Nylander, S. *et al.* Human target validation of phosphoinositide 3-kinase (PI3K)beta: effects on platelets and insulin sensitivity, using AZD6482 a novel PI3Kbeta inhibitor. *Journal of thrombosis and haemostasis : JTH* **10**, 2127-2136, doi:10.1111/j.1538-7836.2012.04898.x (2012).

325 Payne, S., De Val, S. & Neal, A. Endothelial-Specific Cre Mouse Models. *Arteriosclerosis, thrombosis, and vascular biology* **38**, 2550-2561, doi:10.1161/atvbaha.118.309669 (2018).

326 Davis, J., Maillet, M., Miano, J. M. & Molkentin, J. D. Lost in transgenesis: a user's guide for genetically manipulating the mouse in cardiac research. *Circulation research* **111**, 761-777, doi:10.1161/circresaha.111.262717 (2012).